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Research progress in the radioprotective effect of superoxide dismutase

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ABSTRACT: Irradiation from diverse sources is ubiquitous and closely associated with human activity. Radiation therapy (RT), an important component of the multiple radiation origins, contributes significantly to oncotherapy by killing tumor cells. On the other hand, RT can also cause some undesired normal tissue injuries that afflict numerous cancer patients. Although many promising radioprotective agents are emerging, few of them have entered the market successfully due to various limitations. At present, the most accepted hypothesis for the radiation-caused injury involves reactive oxygen species (ROS) generation. Superoxide dismutase (SOD), the unique enzyme responsible for the dismutation of superoxide radicals, is expected to occupy an indispensable position in the treatment of ROS-mediated tissue injuries originating from exposure to radiation. This review focuses on the mechanism of radioprotection by SOD at the tissue or organ level, cellular level, and molecular level, respectively, in order to provide references for further investigation of radiation injury and development of new radioprotectors.

Keywords: Superoxide dismutase, radioprotection, radiation, reactive oxygen species

1. Introduction

Irradiation from diverse sources is ubiquitous and closely associated with human activity. As shown in Figure 1 (*1*), among all sources of radiation, natural radiation, including radon, thoron, cosmic radiation, and natural radioactivity in soils and food, dominates in the average doses of individual radiation adsorption. However, the dose from natural radiation is not under human control. By contrast, artificial radiation, which consists primarily of medical exposure of patients, accounts for approximately 14 percent of the individual radiation absorption and has been attracting more and more attention over recent years. Moderate application of man-made radiation is extremely beneficial as shown by the excellent performance of radiation therapy in oncology (*2*). However, some adverse effects accompany the therapeutic benefit as a consequence of the unavoidable exposure of the surrounding normal tissues to radiation. In this sense, besides the skin being irradiated directly, other internal radiosensitive organs also cannot escape from being injured to different degrees (*3*), which become an impediment, counteracting the efficacy of radiation therapy (RT). In order to minimize these undesired side effects, many efforts have been made to improve the RT technology, such as image-guided radiotherapy, proton radiotherapy, and intensity-modulated radiotherapy. Even though these advanced techniques have the advantage of improved accuracy and control of irradiation, the patients still confront the potential risk of normal tissue injuries (*2*). Thus, to seek the radiation modifiers with selective protection for normal tissues has been a realm of intense investigation.

Despite the fact that many promising radioprotective candidates are emerging, amifostine (WR2721) is the only one approved for clinical use to date. In addition to its high efficacy in ameliorating xerostomia resulting from irradiation (*4,5*), high frequencies of deleterious

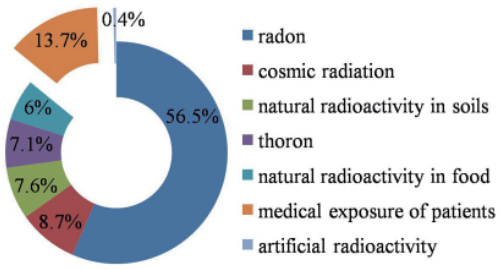

Figure 1. Dose contribution to the individual radiation absorption from all sources of radiation.

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side effects (nausea, cutaneous reactions, hypotension, *etc*.) and even tumor radioprotection have been reported, which limit its use (*6-9*). Therefore, the search for other radioprotectors with high potency and low toxicity should be the primary subject of further research.

At present, various compounds are being evaluated and examined for their radioprotective properties. It has been reported that ReIB, one of the five NF-κB family members existing in mammals, can improve the radioresistance of prostate cancer cells through up-regulation of the mitochondria-localized manganese-superoxide dismutase (Mn-SOD) expression (*10*). Meanwhile, Murley *et al.* have carried out a series of research on the delayed radioprotection for RKO36 cells (a strain of human colon carcinoma cells). They found that preincubation of RKO36 cells with WR1065 (the free thiol form of amifostine) or tumor necrosis factor alpha (TNF-α) could effectively stimulate the expression of Mn-SOD, thereby enhancing the adaptive response of the cells to the subsequent radiation challenge (*11,12*). Additionally, in the study by Zhang *et al*., CpG-oligodeoxynucleotide (CpG-ODN) was shown to effectively relieve bone marrow hemopoiesis radiation injury. Interestingly, the mechanism by which CpG-ODN acted was also through activating the NF-κB pathway and elevating Mn-SOD content (*13*).

These several series of evidence indicate that SOD plays a significant role in radioprotection, and it is of great importance to investigate further details of its mechanism of action so as to develop it as a radioprotector. This review describes the radioprotective studies of SOD based on the hypothesis of reactive oxygen species (ROS) generation associated with radiation injury.

2. Reactive oxygen species and radiation injury

Reactive oxygen species (ROS), *in vivo* byproducts of oxygen metabolism, comprise a multitude of family members such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical (·OH) and so on (*14*). Though the physiological levels of ROS are critical for a variety of cellular functions, such as cell growth, stress adaption, injury response, and cellular phenotype development (*15*), inevitable toxicities can be induced by overdoses of ROS under some pathological conditions (*16*). Homeostasis of ROS is not only affected by endogenous factors but also by exogenous ones (Table 1).

Table 1. Summary of diverse sources of ROS

Endogenous sources	Exogenous sources
NAD(P)H oxidase	Radiations
Xanthine oxidase	Pathogens
P-450 monooxygenases	Metals: Fe, Cu, Zn
Lipoxygenases	Xenobiotics, *etc.*
Cyclooxygenases, *etc.*	

Note: This table summarizes the diverse sources of ROS based on the authors' interpretation of the references (*14*) and (*17*). Readers can refer to the web version of these articles.

Ionizing radiation (IR), possessing great strength of penetration, usually exerts its harmful effects on organisms and biomolecules through both direct and indirect effects. The former is referred to the irreversible injuries caused by radiation selectively impacting certain biomolecules, of which DNA damage is the most notable one. The latter is associated with the condition, in which radiation interacts with non-targeted molecules, induces abnormal levels of ROS exceeding the capacity of the organism to clear them, and, consequently, leads to oxidative stress-mediated damaging effects (*2*). Owing to the highly oxidative activity, ·OH, the radiolysis product of water, contributes a lot to the adverse reactions immediately after radiation by breaking chemical bonds and promoting lipid peroxidation. This explains the significant role of water in the indirect effects of IR (*18*). Moreover, it has been demonstrated that the radiation-exposed organisms perpetuate elevated levels of ROS (*19-21*), caused to a large extent by the mitochondrial dysfunction. Under such condition, molecular oxygen (O_2) is partly reduced to generate considerable amounts of $O_2^{\cdot-}$ and H_2O_2 (*22*). Then, by the way of Fenton and Haber-Weiss reactions, respectively, both $O_2^{\cdot-}$ and H_2O_2 can be further converted to ·OH (*23*), which is the most toxic of all ROS responsible for the majority of IR-mediated adverse reactions. Depending on the photochemical reaction between radiation and some endogenous photosensitizers localized in the cellular or mitochondrial membrane, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (*24*), huge doses of ROS are generated, overwhelming the antioxidant defense system in organisms, which result in a series of serious adverse effects.

Depending upon the level of injury, the IR-induced damages are usually categorized as follows: tissue or organ level effects, cellular level effects and molecular level effects (see Figure 2).

3. Superoxide dismutase and its radioprotective effects

Approximately eighty years ago, Keilin *et al.* isolated a blue protein containing copper from bovine erythrocytes for the first time and named it ergthrocuprein. However, they had no idea about its bioactivity. Not until 1969 did

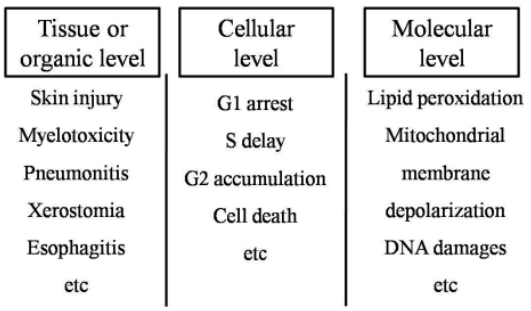

Figure 2. IR-induced damaging effects at different levels.

McCord and Fridorich discover that this protein has the enzymatic activity of catalyzing $O_2^{\cdot-}$ dismutation and formally denominate it superoxide dismutase (SOD) (*25*), which is regarded as a thumping breakthrough that triggered extensive research on SOD from then on.

As naturally present antioxidant enzymes, SODs exist in mammals in diverse forms. There are Cu, Zn-SOD in cytoplasm and nucleus, Mn-SOD oriented in mitochondria, and EC-SOD present mainly in extracellular spaces (*25-27*). Their three-dimensional structures are depicted in Figure 3.

All of the SODs can accelerate the dismutation of $O_2^{\cdot-}$ with a powerful potency to yield O_2 and H_2O_2; the latter is further decomposed into nontoxic products by catalase (CAT) or glutathione peroxidase (GPX). Besides the conventional enzymes mentioned above, some other catalysts such as thioredoxins (TRXs) and glutaredoxins (GRXs) can also facilitate the degradation of H_2O_2 *via* modulating the redox balance of disulfides (see Figure 4) (*28*). Hence, a naturally occurring antioxidant enzymatic defense system is established in the organism involving SOD, CAT, GPX, TRXs, GRXs, and so on.

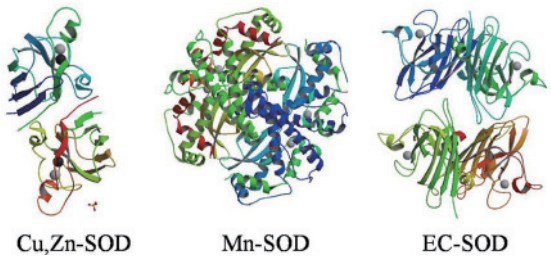

Figure 3. Structures of different SODs in mammals from the Protein Data Bank (PDB). A, Human Cu, Zn-SOD (PDB ID 1PU0); **B**, Human Mn-SOD (PDB ID 1EM1); **C**, Human EC-SOD (PDB ID 2JLP). Human Cu, Zn-SOD is a homodimer with a molecular weight of 32 kDa. Each monomer has one Cu and Zn acting as their active center. Containing the same metal ions as Cu, Zn-SOD, human EC-SOD is a homotetramer with a molecular weight of approximately 135 kDa. Human Mn-SOD is also a homotetramer with molecular weights ranging from 86 to 88 kDa. Distinctively, Mn is essential for its enzymatic activity.

With the growing interest in SOD, it has become clear that SOD acts fundamentally for defeating ROS-mediated diseases such as carcinoma, inflammation and aging (*29,30*). However, these diseases are beyond the scope of this review. Herein, we focus on the protective effects of SOD against IR-induced normal tissue injury at the tissue or organ level, cellular level, and molecular level, respectively.

3.1. *Radioprotection at the tissue or organ level*

Generally, large doses of radiation can lead to a remarkable reduction of parenchymal cells and development of tissue fibrosis, while the vessel wall of mesenchyma becomes thickened and microcirculation gets blocked. All of these together may provoke the burst of organ dysfunction eventually, if not repaired promptly (*31*). However, due to the dissimilar radiosensitivity, different tissues or organs show marked diversity in their responses to the radiation.

Skin, the firstly affected target of radiation, is one of the most acknowledged tissues with respect to the IR-induced injury. During the early period following the

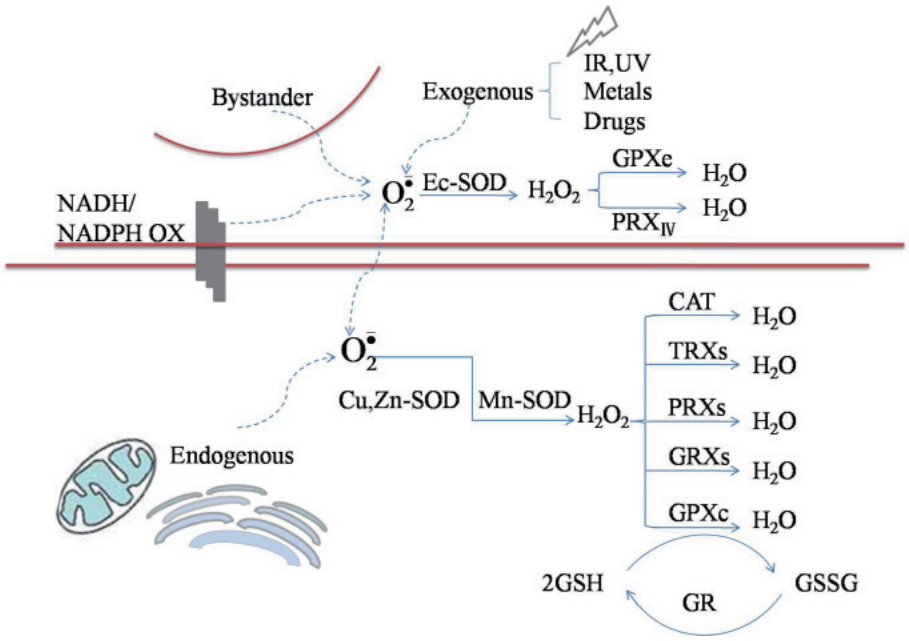

Figure 4. The antioxidant enzymatic defense mechanism naturally occurring in organism. CAT: catalase; GPXc: classic (intracellular) glutathione peroxidase; GPXe: extracellular glutathione peroxidase; GSH: reduced glutathione; GSSG: oxidized glutathione; GR: glutathione reductase; GRXs: glutaredoxins; PRXs: peroxiredoxins (thioredoxin peroxidase); PRX_{IV}: peroxiredoxin IV; TRXs: thioredoxins; NADH/NADPH OX: nicotinamide adenine dinucleotide reduced/nicotinamide adenine dinucleotide phosphate reduced oxidases; IR: ionizing radiation; UV: ultraviolet.

radiation, it is common to observe the appearance of local hyperemia and erythema on skin surface. Thereafter, owing to the long-term, persistent and epigenetic effects of radiation, the lesion may evolve into a chronic skin injury which has no efficient treatment (*31*). In the study by Yan *et al.*, after a single dose of 37 Gy was given to mice on their right hind legs, AAV2-Mn-SOD-hrGFP, a recombinant adeno-associated virus vector expressing Mn-SOD tagged with humanized recombinant green fluorescent protein, was injected subcutaneously in the experimental group and an equal volume of AAV2-IRES (internal ribosome entry site)-hrGFP in the control group. Although all the irradiated mice demonstrated severe skin injury initially, the mice in the experimental group displayed pronounced mitigation and accelerated healing process ($p < 0.05$) two weeks after radiation compared with the control group. These results indicate the marked relief of IR-induced skin injury provided by Mn-SOD expression (*32*).

Because of the high radiosensitivity, it is obvious that hematopoietic cell proliferation can be suppressed upon the exposure to radiation (*33*). Numerous studies have been performed to explore the role of SOD in the adaptive response of the hematopoietic organ to the radiation. It was observed that intravenous injection of bovine SOD to mice could significantly promote the recovery of erythrocytes, reticulocytes and white blood cells from X-irradiation-induced loss (*34,35*). Moreover, Eastgate *et al.* verified that interleukin-1 (IL-1) administration was able to provide radioprotection of the irradiated mice from myelotoxicity as well as the explanted murine bone marrow cells from the damaging effects of IR mainly due to the effect of increased Mn-SOD expression triggered by IL-1 treatment (*36*).

Lung, another organ vulnerable to radiation damage, is subjected to injury regularly during the radiotherapy of patients with lung carcinoma or other thoracic cancers. Depending on the time of onset of lung injury as well as the pathological features exhibited, there are two kinds of IR-induced lung injury: acute radiation pneumonitis manifesting exudative inflammation and interstitial edema, and lung fibrosis characterized by the thickening of alveolar wall due to long-time exposure to radiation (*37*). However, these two types of lung injury are thought to be independent (*38*). Provided that lung fibrosis arising from radiation was a result of a cascade of cytokines among which TGF-β1 was a critical one (*39*), Machtay *et al.* used RT-PCR to detect the level of TGF-β1 expression in mouse lung homogenate and demonstrated that the group of mice treated with PEG-AOE (PEG-antioxidant enzyme composed of PEG-SOD and PEG-CAT at the ratio of 1:1) showed a notably diminished level of TGF-β1 compared with the group that received irradiation alone. In addition, further analysis indicated a remarkable reduction in the lung hydroxyproline content by PEG-AOE administration (*40*). The data above supports the concept that PEG-AOE has a great potential to reverse radiation-induced lung fibrosis.

For the head-and-neck cancer patients, local radiotherapy always results in xerostomia and oral mucositis (*41*), which bring them serious discomfort. By means of flow cytometry, it was found that the sharp reduction of salivary secretion in the mice irradiated at head-and-neck region was caused by the overproduction of ROS resulting from radiation. However, this phenomenon was not observed in the group of mice with PC-SOD (lecithinized SOD) treatment due to the capability of PC-SOD to scavenge $O_2^{\cdot-}$ during the whole experimental process (*42*). Subsequently, another study by Nagler *et al.* showed a similar trend in the Wistar rats administered with Mn-SOD, which showed a dramatic resistance against hyposalivation induced by local head-and-neck radiation (*43*). Collectively, these findings suggest that SOD can effectively protect the saliva gland from radiation injury and neutralize IR-mediated hyposalivation.

Esophagitis is a major complication developed in the non-small cell lung cancer patients receiving RT. Stickle *et al.* found that intraesophageal injection of Mn-SOD-PL (plasmid liposome) prior to radiation could significantly prevent the development of vacuole in the esophageal squamous lining cells ($p < 0.001$) and elevate the mouse survival ($p = 0.0009$) suggesting the protective effect provided by Mn-SOD-PL-mediated SOD expression against esophagitis (*44*).

3.2. *Radioprotection at the cellular level*

It is well accepted that cell cycle is susceptible to radiation which can induce G1 arrest, S delay, and G2 accumulation (*45*). Nonetheless, attributable to the long-time evolution, the cell has developed a series of cell cycle checkpoints including G1/S checkpoint, S checkpoint, and G2/M checkpoint. All the checkpoints above collaborate to initiate related repair mechanisms and to guarantee the normal transition from one phase to another depending upon the activity of cell phase-specific cyclins and cyclin-dependent protein kinases (CDKs). A representative profile for the regulation of mammalian cell cycle is described in Figure 5 *(46)*.

During S phase, the cell needs to absorb appropriate amounts of ribonucleosides to maintain nucleic acid synthesis. In the study by Epperly *et al.*, 5-bromo-2-deoxyuridine (BuDR) was given to the pre-irradiated mice by intraperitoneal injection and the intake of BuDR by oral cavity mucosal cells was measured one hour later to estimate the state of S phase. It was found that the groups of mice irradiated or irradiated with WR2721 administration alone showed BuDR intake multiple times higher than the normal control. However, this phenomenon was suppressed significantly in the mice treated with Mn-SOD-PL alone or Mn-SOD-PL combined with WR2721, which verified the hypothesis that SOD could suppress the radiation-induced G1 arrest to exert its protective effects (*47*).

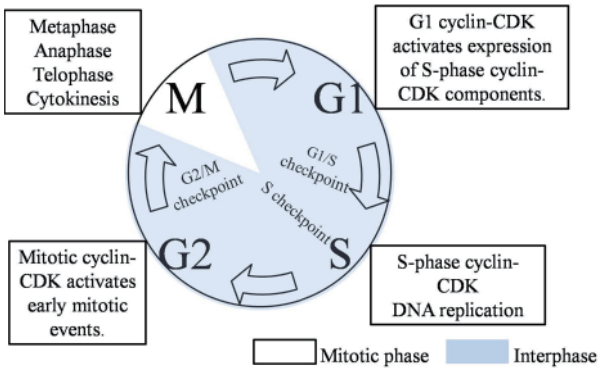

Figure 5. Regulation of mammalian cell cycle. G1: 1st gap; S: DNA synthesis phase; G2: 2nd gap; M: mitosis and cytokinesis. As shown in this figure, cell cycle consists of mitotic phases and interphases which are further classified into G1, S, and G2. G1 is also called presynthetic phase during which DNA pre-replication complexes accumulate and prepare for DNA synthesis. S is regarded as the core of interphase. In this phase, DNA is replicated adequately to enable the final cell division. G2 is referred to post-synthetic phase, during which DNA replication is completed and some early mitotic events are activated to be ready for the entry to M phase. M comprises metaphase, anaphase, telophase and cytokinesis. After separation of both chromosomes and cytoplasm, two new daughter cells carrying the same genetic material as that of the parent cell are obtained. Obviously, the ordered transition among different phases cannot do without the assistance of corresponding cyclins specific for every phase. It is these cyclins that act as the key regulatory factor in respective checkpoint.

G2/M checkpoint is devoted to prohibit the cell with damaged DNA from entering M phase directly. In particular, compound Cdc2(Chk1)-CyclinB involved in the transition from G2 to M is inactivated either by ATM-Chk2-Cdc25 or ATR-Chk1-Cdc25 pathway (*48*). Gao *et al*. showed that radiation induced considerable DNA damage and apoptosis as measured by the large increase of the percentage of cells with sub-G1 content. Yet, this outcome was not observed in the irradiated cells overexpressing Cu, Zn-SOD, where the protein CyclinB1 content decreased by 60-70% compared with the control and the percentage of cells accumulated in G2 phase increased significantly. The results demonstrate that Cu, Zn-SOD is able to provide radioprotection by the way of down-regulating CyclinB1 activity, retarding the G2/M transition and promoting DNA repair (*46*). Additionally, in accordance with the finding above, Kalen *et al.* obtained a similar conclusion that Mn-SOD could efficiently initiate the G2/M checkpoint to produce cytoprotection (*49*).

On the other hand, SOD is envisioned to perform its cellular level radioprotection through suppressing the abnormal proliferation. It is well accepted that stem cells usually multiply slowly to make the exact response to the signal from the external environment and determine whether to proliferate directly or differentiate for the sake of reducing DNA mutation and preventing tumorigenesis (*50*). Take the mouse esophageal side population (SP) stem cells as an example: samples from the mice irradiated alone showed a significant increase in the content of PCNA (proliferating cell nuclear antigen) compared to the background ($p < 0.0001$), while in contrast, the esophageal SP stem cells from mice given Mn-SOD-PL kept the PCNA level commensurate to the normal control without any perturbation of multi-direction differentiation capacity, which confirms the theory described above regarding the action of SOD (*51*).

Clearly, the cellular level radioprotection provided by SOD can be ascribed to its capacity of either regulating the cell cycle checkpoint or inhibiting the abnormal proliferation of the cell. Therefore, it is concluded that SOD is able to act as a promising radioprotector to maintain the steady-state of the cell proliferation and restrict the inclination to carcinogenesis.

3.3. *Radioprotection at the molecular level*

As described previously, based on the interaction between radiation and *in vivo* biomolecules, excessive ROS are generated, which lead to the various pathological symptoms, implicating the significance of the molecular level radioprotection conducted by SOD.

Through direct interaction with radiation, lipids, the major constituents included in the construction of biomembrane, can be oxidized to produce considerable peroxidized lipids and malonaldehyde (MDA), which pose a threat to the integrity of the membrane structure (*52*). Early in 1976, a related study was conducted to determine the role of SOD involved in preserving the phospholipid biomembrane *in vitro* when it was exposed to radiation. The data revealed a notable increase in the amount of peroxidized lipids in the irradiated biomembrane as evidenced by the increased absorbance at 232 nm. Adversely, the same phenomenon was not observed in the biomembrane pre-incubated with bovine SOD at an extremely low concentration of 1 ng/mL, demonstrating the striking protection effect of SOD on biomembrane *in vitro* against lipid peroxidization caused by radiation (*53*). Recently, Epperly *et al.* used the irradiated mouse model transfected with Mn-SOD-PL to verify the hypothesis that the *in vivo* lipid peroxidization was partly regulated by the cytokines involved in the cell division to substitute the injured cells. By means of RNase protection assay, a detectable up-regulation of cytokines such as IFNγ and TNFγ was observed in the irradiated control mice but not in the mice with Mn-SOD-PL administration. Consistent with that, the latter mice also showed relatively lower level of peroxidized lipids after receiving radiation as compared with the irradiated control value, which confirmed their hypothesis successfully (*54*).

In addition, it is also worthwhile to mention the SOD-initiated radioprotective effect on mitochondria, the essential organelle in which membrane depolarization is responsible for numerous detrimental reactions such as the release of cytochrome *c*, the activation of caspase, the uncoupling of oxidative phosphorylation (*55*). Over the past years, it has been proved that overexpression of Mn-SOD in irradiated cells significantly decreased the

occurrence of mitochondrial membrane depolarization which was commonly seen in the control group (*56*). Besides, when Gorman *et al.* investigated the bystander effect of radiation, a remarkable genomic instability coupled with mitochondrial membrane depolarization was observed in the bystander cells. However, after transfection with Mn-SOD-PL, the biological reactions above were significantly inhibited. These results demonstrate the efficient radioprotection by SOD on mitochondria (*57*).

Similarly, DNA also seems to be the target biomolecule of radiation. It is well established that after radiation, a broad range of DNA damages are induced such as base damage, single strand breaks (SSBs), and double strand breaks (DSBs) if initial damage on DNA is not repaired properly. Among them, DSBs is regarded as the most deleterious one for its ability to arouse homologous recombination (HR) (*58-61*). Then, HR can further generate base insertion, depletion, translocation along with high carcinogenicity (*59-61*). Peroxynitrite, the product of the reaction between nitrogen monoxidum and superoxide, is able to trigger the formation of genomic rearrangement directly, indicating the key role of ROS scavenging in blocking cancer generation (see Figure 6). In the study by Niu *et al.*, fluorescent yellow direct repeat (FYDR) mice were employed to estimate the incidence of HR *in vivo* measured by the number of fluorescent recombinant cells using flow cytometry. The data showed a significant increase in fluorescent recombinant esophageal cells in the irradiated FYDR mice compared to the control, which indicated that a large degree of HR was stimulated by radiation. Whereas, in the irradiated FYDR mice with Mn-SOD-PL administration, the level of fluorescent recombinant cell counts was almost near the normal value (*62*). This study provides a powerful evidence for the potential of SOD to prevent HR induced by radiation. Furthermore, using agarose gel electrophoresis, Liu *et al.* found that the radiation-induced plasmid DNA damages, such as the increased amount of open circular, could be effectively suppressed by the pre-incubation of Hep-SOD (heparin-SOD conjugate) *in vitro* (*63*).

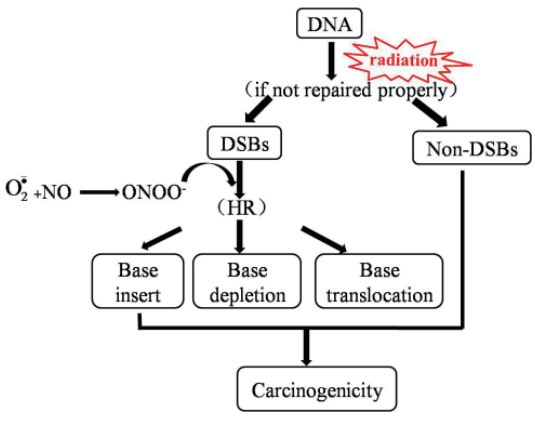

Figure 6. DNA damages triggered by radiation. HR: homologous recombination; DSBs: double strand breaks; Non-DSBs: single strand breaks, translocation, base damage, *etc*.

4. Conclusion

As the unique enzyme capable of dismutating $O_2^{\cdot-}$, SOD is expected to occupy an indispensable position in the treatment of ROS-mediated normal tissue injuries originating from exposure to radiation. Although SOD-related antiradiation research has been continued for nearly forty years and many positive outcomes have been obtained, hardly any drug based on SOD has been approved for radioprotective use in the clinic. Up to now, Orgotein is the only SOD product used as a radioprotector mainly in animals because of its inclination to induce allergic reaction in human (*64*). Other factors restricting its entry into clinical treatment include its large molecular weight, inability to pass the cell membrane freely, short half-life (*65*), rapid metabolic rate, narrow time-window of action (*66*) and so on. To solve these problems, scientific community has resorted to the investigation of SOD-based gene therapy, SOD conjugates, and non-enzymatic SOD mimics.

SOD-based gene therapy mediated by the plasmid liposome or recombinant virus vector showed positive outcomes in numerous research studies as described in this paper. This strategy addresses the poor membrane permeability and low expression of native SOD found in organisms. Additional work needs to be done to explore the availability of SOD-based gene therapy in human subjects. SOD conjugates obtained through the way of chemical modification have advantages of prolonged half-life, improved cell membrane permeability, augmented bioactivities and efficient targeting compared with the native SOD. An outstanding example of these compounds is Hep-SOD which has been verified for its superior radioprotection owing to its long half-life, and enhanced tolerance to high temperature, strong acid/base, and enzymolysis of trypsin (*67,68*). Besides these advanced forms of SOD, non-enzymatic SOD mimics have also become a favorite form of radioprotective agent to some researchers in recent years. This class of synthetic low molecular-weight compounds containing a metal ion as the active center also shows prolonged half-lives and widened time-windows compared to native SOD. Among them, M40403 (a manganese (II) complex with a bis (cyclo-hexylpyridine) substituted macrocyclic ligand) has been approved by FDA as a radioprotector for cancer patients (*69*).

From the discussion above, we firmly believe the great potential of SOD-based compounds to be developed as novel radioprotectors in the future. At present, the most important task is to continue studying further their pharmacokinetics, toxicity, optimal route of administration and to strive for their radioprotective application in the clinic as soon as possible.

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Review

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An overview on antiepileptic drugs

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ABSTRACT: Epilepsy is the most common chronic neurological disorder of the brain. For several decades different kinds of medications have been used to treat epilepsy. Even though many surgical advances has been made and implemented, medications remain the basis of treatment. The search for noble antiepileptic drugs (AEDs) with more selective activity and lower toxicity continues to be an area of intensive investigation in medicinal chemistry. Additionally, drug resistance is an important clinical problem in epilepsy and is associated with an increased risk of morbidity and mortality. This review intends to present a comprehensive overview on AED in particular along with discussion on some aspects of associated drug resistance and combination therapy.

Keywords: Epilepsy, anticonvulsants, resistance, combination therapy

1. Introduction

Epilepsy is a chronic neurological disorder in which clusters of nerve cells, or neurons, in the brain sometimes signal abnormally that may remain localized (focal epilepsy) or become widespread (generalized epilepsy). The term epilepsy is derived from the Greek word epilepsia, which means "falling sickness" and can be called "seizure", "ictus", or "convulsion" (*1*). Both the electrical and the behavioral aspect of seizures can be quite variable and complex, even in a single patient. Seizures can be caused by a variety of pathologic conditions, including acquired injuries and genetic abnormalities. In addition, many physiologic disturbances of brain function can produce seizures and the prevalence of epilepsy varies from adults to children (*2*). Three to five percent of the population has a seizure sometime in their life and half to one per cent of the population have 'active epilepsy'. The heterogenicity of epilepsy makes it difficult to establish precise epidemiological statistics (*3*).

Etiology of epilepsy. Approximately 60% of all epilepsies are idiopathic. Almost any type of brain pathology can cause seizures/epilepsy. The underlying etiology is numerous and the abnormalities may range from symptomatic seizures due to tumor, infection, and trauma to cryptogenic forms. Cerebrovascular disease is the most commonly identified cause among adults, while prenatal insults seem to be most common among children (*4*). An imbalance between glutamate and γ-aminobutyric acid (GABA) neurotransmitter systems can lead to hyperexcitability. Catecholaminergic neurotransmitter systems and opioid peptides are also shown to play a role in epileptogenesis (*5*). Enhancement of excitatory transmission and simultaneous failure of inhibitory mechanisms together with changes in intrinsic neuronal properties results in repetitive neuronal discharges (*6*). Additionally, innate immunity/inflammation, adaptive immune responses, and inflammation markers including autoantibodies also play a role in the pathophysiology of several types of epilepsy (*7*).

Classification. Classification of epilepsy is the foundation for treatment. Several neuropsychiatrists with a special interest in epilepsy put forward the idea that the existing systems of classification of psychiatric disorders and personality disorders are inadequate as far as epilepsy is concerned. Classification of seizure type is dependent on the accuracy of history, availability and sophistication of diagnostic tests used, and age at which the patient's seizure type was classified. International Classification of Epileptic Seizures System (ICES) was introduced in 1970 and revised in 1981. Generally seizures can be classified as partial, generalized, and unclassified ones. The Commission on classification and terminology of the International League Against Epilepsy (ILAE) recently recommended new terminology and concepts on focal and generalized epilepsy. According to etiologic classification, *viz.*, the idiopathic, symptomatic, and cryptogenic forms of epilepsy have been conceptualized as genetic, structural/metabolic, and unknown forms of epilepsy (*8*). A general classification of epilepsy is shown in Figure 1.

Global burden. The WHO global burden of disease (GBD) 2004 update estimates that about 40 million individuals globally have epilepsy. Inclusion of epilepsy

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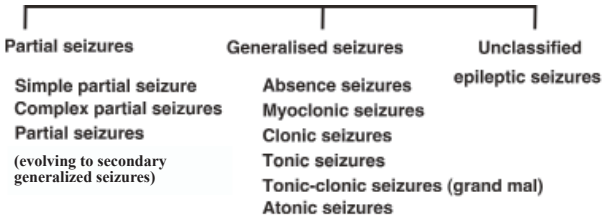

Figure 1. The classification of epilepsy.

caused by other disease or injury may increase the total number of persons affected in the world to about 50 million (*9*). The incidence of epilepsy in developed countries is between 40-70/100,000/year and the ratio is much higher (120/100,000/year) in resource poor countries (*10*).

Medication. An appropriate diagnosis together with proper selection and utilization of currently available antiepileptic drugs (AEDs) is necessary for therapeutic success in the management of epilepsy. With the range of drugs currently available, there are immense opportunities for patient-tailored drug therapy. However the management of epilepsy is primarily based on optimum use of AEDs with the choice of drugs varying considerably among physicians and across countries. The choice is primarily based on evidence of efficacy and effectiveness for the individual's seizure type, but other patient-specific factors, including age, sex, childbearing potential, adverse-effect profile, comorbidities, and concomitant medications are also needed to be considered (*11*). Further, better understanding of pharmacoresistance would help to replace the current empiricism with a more patient-centric approach towards the management of epilepsy.

2. Antiepileptic drugs (structures see Figure 2)

Bromides were the first medication introduced by Sir Charles Locock in 1857 to provide control for seizures. Bromides were rendered obsolete due to their side effects and are being replaced by newer therapeutics. However, it has now been re-established as an add-on therapy in some selected cases of intractable generalized tonic clonic seizure (*12*). Phenobarbital (PB) (1912) (**1**), a member of the barbiturate class serendipitously discovered by Alfred Hauptmann was as effective as bromides with a less toxic profile, is easier to administer and subsequently replaced the bromides. Primidone (**2**) is another member of the same family whose mechanism of antiepileptic action is not known. Primidone *per se* has anticonvulsant activity as do its two metabolites *viz.*, PB and phenylethylmalonamide (PEMA) (**3**). In the 1930s, the introduction of sulfanilamide was a major medical advance, but in 1937 a sulfanilamide preparation containing diethylene glycol was one of the most consequential mass poisonings of the 20th century known as the elixir sulfanilamide disaster. This led to new regulations for the preparation, safety, testing, labeling, distribution, and marketing of drugs. Hydantoin (glycolylurea) was first isolated in 1861. The precise mechanisms by which hydantoins work are unknown, but they are thought to exert their therapeutic effect by depressing abnormal neuronal discharges in the central nervous system (CNS). The hydantoins include phenytoin (Dilantin®) (**4**) and mephenytoin (Mesantoin®). Phenytoin (5,5-diphenylhydantoin, dilantin; 1938) known as the 'miracle' drug of its day was discovered by H. Houston Merritt and Tracy Putnam. This drug was used as a first choice, or when phenobarbital failed. It was one of the most widely used drugs, effective in tonic-clonic and partial seizures. An unknown substance positive to ninhydrin was found in 1949 by Roberts and Frankel in chromatographed fresh human brain tissue, which was later identified as GABA, the inhibitory neurotransmitter (*13*). The discovery of valproate's effectiveness as an AED created a new therapeutic paradigm. This drug was thought to be effective in enhancing GABA in the nervous system, and became one of the first drugs in which a mode of action was proposed. This drug has been licensed in the UK for clinical use since 1973 and in the USA since 1978. Its own metabolism may be enhanced by other anti-epileptic agents. In the 1970s and 1980s, the AEDs most frequently used to prevent seizures were phenobarbital, phenytoin, and carbamazepine. Later on they were found to cause major malformations, microcephaly, growth retardation, and distinctive minor abnormalities of the face and fingers in infants exposed to them during pregnancy. Gabapentin (**5**) (Neurontin®), marketed in the U.S. in 1993, was the first approved AED not metabolized in the liver, making drug interactions a lesser problem. The mode of action of gabapentin is largely unknown (*14*). Parker *et al.* (2004) (*15*) demonstrated that gabapentin selectively activates presynaptic $GABA_B$ heteroreceptors. Recent studies suggest that it interacts with an auxiliary α2β subunit of voltage-sensitive calcium channels and inhibits the calcium currents leading in turn to reduced neurotransmitter release and attenuation of postsynaptic excitability (*16*). Carbamazepine (Tegretol®) (**6**) is considered as a drug of choice for tonic clonic seizures, partial seizures, and trigeminal neuralgia. It works by decreasing nerve impulses that cause seizures and pain. Succinimides such as ethosuximide (Zarontin®) (**7**) and methsuccimide (Celontin®) are also widely used for absence (petitmal epilepsy) seizures. Another class of compounds that are widely used in the management of epilepsy is benzodiazepines (BDZs). Clinical advantages of these drugs include rapid onset of action, high efficacy rates and minimal toxicity. Among the approximately 35 BZDs available, clorazepate (Tranxene®) (**8**) has a distinctive and favorable profile that includes a long half-life of its active metabolite and slow onset of tolerance (*17*). Standard AEDs usually

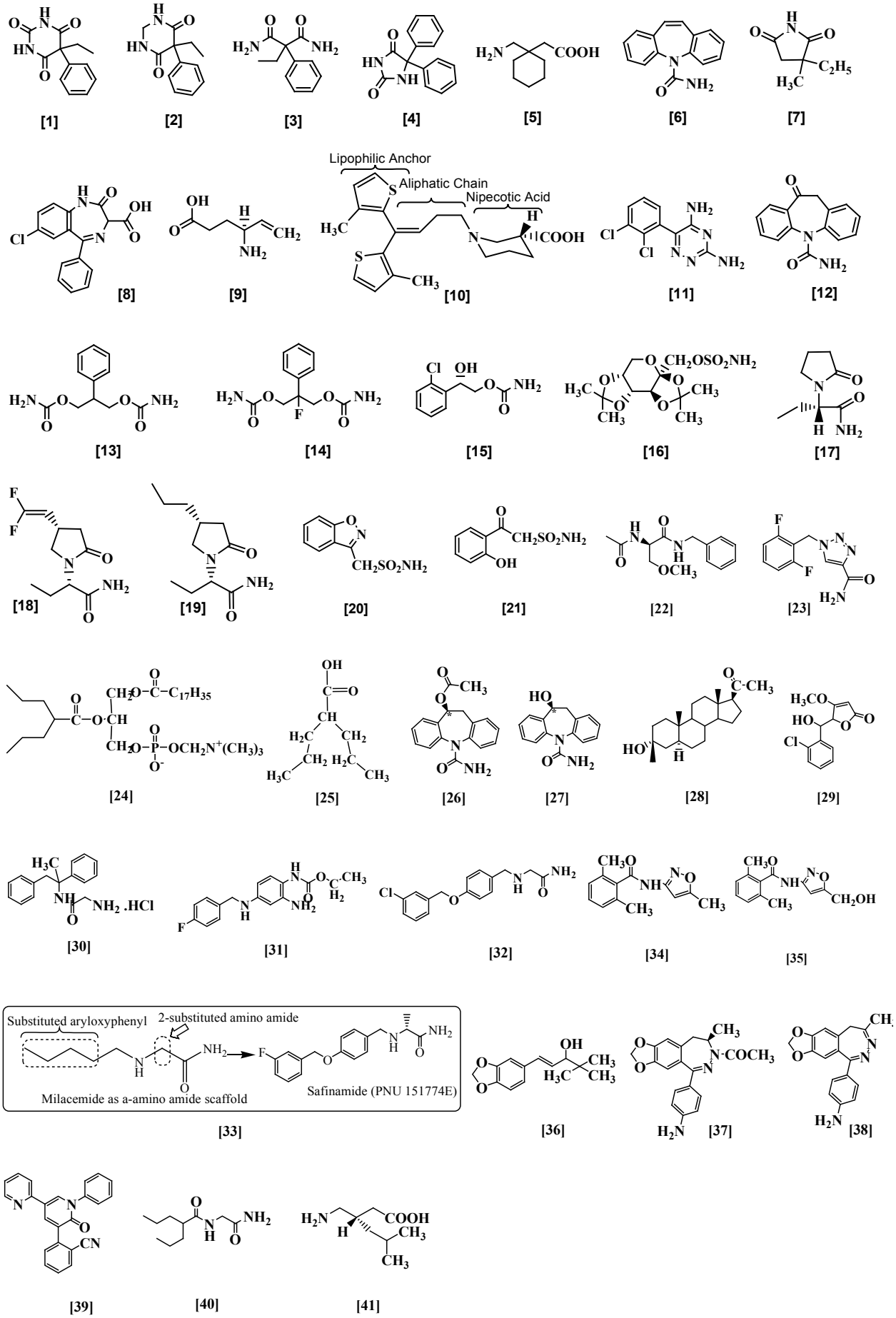

Figure 2. Chemical structures of antiepileptic drugs.

produce side effects in 50% or more of patients treated. Rare but serious idiosyncratic reactions were reported which include agranulocytosis, Stevens-Johnson syndrome, aplastic anaemia, hepatic failure, allergic dermatitis, serum sickness, and pancreatitis (*18*). Once the seizure type and epilepsy syndrome have been determined, an AED can be appropriately selected. During the past decade, a number of new AEDs have been developed with diverse mechanism of action (Table 1). Most of the AEDs are efficacious for partial-onset seizures and were originally approved based on their efficacy as add-on therapy in patients with refractory partial-onset seizures. For patients with generalized-onset seizures the choice of therapy is narrower and includes valproate as well as the newer agents such as lamotrigine and topiramate. From the last 30 years many newer drugs were introduced with better safety profiles. The aim of epilepsy therapy is to keep the patient free of seizures without interfering with normal brain function. The currently available armamentarium of AEDs is discussed below.

*Vigabatrin (γ-vinyl GABA, Sabril®) (**9**).* Vigabatrin (VGB), a structural analogue of GABA possesses a vinyl appendage. It irreversibly inhibits GABA-transaminase (GABA-T), the enzyme responsible for the catabolism of GABA, thereby increasing the whole-brain levels of GABA making it more available to its receptor site (*19*). Thus VGB acts as an indirect GABA agonist. It has emerged as a first choice AED in the treatment of refractory epilepsies such as infantile spasms (also called WEST syndrome), particularly those accompanied by tuberous sclerosis (*20*). Long term vigabatrin treatment is associated with persistent visual field problems that have led to a reduction in the use of the drug (*21*). In order to minimize the chance of visual field defects, the treatment of infantile spasm may be started for brief periods at very early onset with low doses of VGB (*22*).

*Tiagabine (**10**).* Tiagabine (TGB) [(*R*)-(−)-1-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]3-piperidine carboxylic acid hydrochloride] is a nipecotic acid analogue, into which a lipophilic anchor has been incorporated to facilitate crossing of the blood-brain barrier after oral administration. It is a highly selective and potent inhibitor of GABA transporter 1 (GAT-1) in presynaptic neurons and glial cells. TGB selectively increases the amount of the inhibitory neurotransmitter GABA at the GABAergic synapse and is the first of its kind to be introduced into clinical practice (*23*). Isobolographic analysis of the interactions of TGB with three conventional AEDs: valproate (VPA), carbamazepine (CBZ), and phenobarbital (PB) in amygdala-kindled rats showed synergistic interaction (fixed-ratio combinations of 1:3, 1:1, and 3:1, respectively). The study suggested that TGB appears to be a valuable drug for an add-on therapy of refractory complex partial seizures in humans (*24*). However, a recent study revealed that the efficacy of tiagabine in newly diagnosed epilepsy is relatively low when prescribed along with other AEDs. A critical side effect such as induction of non-convulsive status epilepticus limits its use (*25*).

*Lamotrigine (Lamictal®) (**11**).* Lamotrigine (LTG), a triazine derivative that inhibits the release of glutamate (an excitatory amino acid) has been approved for use as an adjunct drug in treatment of refractory partial seizure

Table 1. Antiepileptic drugs and their mechanism of action

Drugs	Mechanism of action
Vigabatrin	Irreversibly inhibits GABA-T
Tiagabine	Inhibitor of GAT-1
^Lamotrigine	Inhibits the release of excitatory neurotransmitter glutamate. It also inhibits the voltage-sensitive Na^+ channels (VDSC); Blockade of α4β2-nAChR
Carbamazepine, oxcarbazepine, eslicarbazepine	Stabilize the inactivated state of VDSC
^Felbamate, fluorofelbamate	Inhibits NMDA receptor. Also potentiates GABA-mediated inhibition and blocks VDSC
Carisbamate, rufinamide, losigamone, soretolide, valrocemide	Yet to be ascertained
^Topiramate	Selectively blocks excitatory synaptic transmission mediated by GluR5 kainate receptors; also acts at VDSC
Levetiracetam, seletracetam, brivaracetam	Interacts with the synaptic vesicle protein 2A
^Zonisamide	Block sodium channels and reduce voltage dependent T-type Ca^{2+} currents; also modulates dopaminergic, GABA ergic, and serotonergic systems
^Lacosamide	Enhances slow inactivation of VDSC and modulates CRMP-2
Ganaxolone	Positive allosteric modulation of the $GABA_A$ receptor
Remacemide	Potent Na^+ channel blocker and non-competitive NMDA channel antagonist.
Retigabine	It is a KCNQ K^+ channel opener that involves opening of neuronal Kv7.2 (KCNQ2) voltage activated K^+ channels
^Safinamide	Antagonize the Ca^{2+} and Na^+ channels; also reversibly inhibit MAO-B
Stiripentol	It is positive allosteric modulator of $GABA_A$ receptor
Talampanel, perampanel	Non-competitively blocks AMPA receptor
Pregabalin	It binds potently to the α2-γ subunit, an auxiliary protein associated with voltage-gated Ca^{2+} channels

^ Indicates multiple mechanisms.

with or without generalized tonic/clonic seizures. It also inhibits the voltage-sensitive sodium channels thereby stabilizing the neuronal membrane (*26*). Recent studies further suggested that the neuronal α4β2-nAChR (neuronal nicotinic acetylcholine receptor) is likely an important target. The blockade of α4β2-nAChR might represent the mechanism through which LTG effectively controls some types of epilepsy such as autosomal dominant nocturnal frontal lobe epilepsy or juvenile myoclonic epilepsy (*27*). Among the adverse effects, idiosyncratic drug reactions, especially skin rashes are considered fatal and may require discontinuation of the drug. The parent drug rather than a reactive metabolite causes LTG-induced skin rashes. Rash is relatively more common in children than in adults and it is safe when used in general practice to treat epilepsy inadequately controlled by other medications. Serious adverse events were rarely reported and included Stevens-Johnson syndrome (*28*). Long term treatment with LTG may cause hepatic inflammation and it has been found that dextran conjugate prodrug has the potential to reduce the hepatotoxicity (*29*). LTG monotherapy was found to be an effective treatment for children with newly diagnosed childhood absence seizures and an extended-release formulation (LTG-XR) may be given once daily for increasing compliance (*30*). Further a randomized, double-blind, placebo-controlled study reported that adjunctive therapy with LTG-XR administered once daily to a target of 200 to 500 mg/day significantly reduced weekly frequency of primary generalized tonic-clonic (PGTC) seizures and increased the percentage of patients with a ≥ 50% reduction in PGTC seizure frequency (*31*). A retrospective population-based study by Knoester *et al*. (2005) suggests that LTG was effective in 40% of the patients with refractory epilepsy measured by reduction in seizure frequency and retention time as observed in 165 patients. The drug is known to have a more favorable side-effect profile than conventional AEDs (*32*). An unblinded randomized controlled trial carried out by the Standard and New Antiepileptic Drugs (SANAD) study group found lamotrigine to be clinically better than CBZ, the standard drug treatment, for time to treatment failure outcomes and is considered as a cost-effective alternative for patients diagnosed with partial onset seizures (*33*).

*Oxcarbazepine (TRILEPTAL®) (**12**).* Oxcarbazepine (OBZ), 10,11-dihydro-10-oxo-5*H*-dibenz[b,f]azepine-5-carboxamide is a prodrug which is activated to eslicarbazepine in the liver. OBZ is primarily used in the treatment of epilepsy. It is also used to treat anxiety and mood disorders and benign motor ticks. OBZ is a structural derivative of CBZ, with a ketone in place of the carbon-carbon double bond on the dibenzazepine ring. This difference helps reduce the impact on the liver of metabolizing the drug, and also prevents the serious forms of anemia or agranulocytosis occasionally associated with CBZ (*34*).

*Felbamate (Felbatol®) (**13**).* Felbamate (FBM) is structurally related to meprobamate. Its activity in epilepsy probably involves effects on the NMDA receptor. It also potentiates GABA-mediated inhibition and blocks voltage-dependent sodium channels. FBM is an effective and safe AED for either monotherapy or add-on treatment in adults with refractory partial seizures. It is also effective and safe for the treatment of refractory Lennox-Gastaut syndrome in both children and adults (*35*).

*Fluorofelbamate (**14**).* Fluorofelbamate (FFBM, 2-phenyl-2-fluoro-1,3-propanediol dicarbamate) is new chemical entity different from FBM in that fluorine is substituted for hydrogen in the two position of the propane. Mazarati *et al*. (2002) (*36*) studied the effectiveness of FFBM using a rat model of self-sustaining status epilepticus (SSSE). They found that FFBM exhibited a much better activity profile including no recurrent seizure activity in aborting SSSE when injected at both its early and advanced stages where diazepam and phenytoin failed to abort SSSE when administered after 40 or 70 min after the onset of stimulation. The drug candidate is designed to retain the activity of felbamate but with a different metabolic pathway that restricts the formation atropaldehyde/acid-glutathione adduct (ATPAL-GSH and ATPA-GSH) the reactive aldehyde and acid metabolite of FBM. Thus fluorofelbamate is devoid of serious idiosyncratic toxicity associated with FBM (*37*). The presence of the fluoro atom protects the amide groups by its inductive effect and does not undergo the formation of ATPAL-GSH and ATPA-GSH (*38*).

*Carisbamate (**15**).* Carisbamate (CBM), or RWJ-333369 ((*S*)-2-*O*-carbamoyl-1-*o*-chlorophenyl-ethanol), is a novel neuromodulator under investigation for the adjunctive treatment of epilepsy. This AED is structurally similar to felbamate. CBM was found to possess a broad spectrum of activity in rodent seizure and epilepsy models. Molecular action that contributes to its broad-spectrum antiepileptic activity is yet to be ascertained (*39*). A study was initiated to investigate mechanisms underlying the antiepileptic effects of carisbamate using the hippocampal neuronal culture models of status epilepticus and spontaneous epileptiform discharges (*40*). CBM has demonstrated antiepileptic activity in a variety of *in vivo* seizure models including hippocampal, corneal kindling, and the Genetic Absence Epilepsy Rats of Strasbourg (GAERS) model of absence epilepsy (*41*). It was also found to be effective in protecting against spontaneous recurrent seizures in kainate-treated animals (*42*) and in genetic models of epilepsy. It delays or prevents the Li-pilocarpine model of status epilepticus (*43*). It is rapidly and almost completely absorbed from the gut with a bioavailability of approximately 95% and with a peak plasma concentration achieved within 1-3 h (*44*).

*Topiramate (Topamax®) (**16**)*. Topiramate (TPM) (2,3:4,5-bis-*O*-(l-methylidene)-*β*-D-fructopyranose sulphamate) is a sulphamate substituted monosaccharide. The specific mechanism of action of TPM is not well understood. Preliminary reports suggested that it has a multiple mode of action. It acts at voltage-dependent sodium channels blocking the spread of seizures, it enhances $GABA_A$ evoked chloride currents at a non-benzodiazepine receptor site and it also antagonizes the 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl) propionic acid (AMPA) subtype of glutamate receptors (*45*). Gryder and Rogawski (2003) (*46*) established that it selectively blocks excitatory synaptic transmission mediated by GluR5 kainate receptors. It is rapidly absorbed with linear pharmacokinetics and in the absence of enzyme inducers *viz.*, phenytoin or carbamazepine has a plasma elimination half-life of 20-30 h. It has relatively low potential for interaction with other AEDs. However during TPM adjunctive therapy with phenytoin, CBZ, and VPA, the minimum plasma concentration level of TPM was reduced to 50%, 40%, and 14%, respectively. In addition, administration of TPM in patients already taking phenytoin twice a day, a 25% rise in the concentrations of phenytoin were found and may require a downward adjustment of phenytoin dosage (*47*). Besides in patients with pharmacoresistant epilepsy, Luna-Tortos *et al*. (2009) (*48*) provided evidence that brain levels of topiramate may be affected by overexpression of P-glycoprotein. Enhanced elimination of TPM was also observed during pregnancy. In particular the plasma concentration of TPM was found to decline approximately by 40% in the 2nd and 3rd trimester (*49*). To evaluate the efficacy of TPM in infants, Grosso *et al*. (2005) (*50*) found that TPM is effective across a broad range of seizure types in infants aged less than 2 years. The study also provides evidence regarding the usefulness of TPM in cryptogenic infantile spasms but it showed poor efficacy in symptomatic infantile spasms and in Dravet's syndrome. A randomized double-blind clinical trial demonstrated that TPM may be an alternative for phenytoin in patients for whom urgent treatment is required. TPM was found to be well tolerated as observed from the lower rate of incidence of adverse effects in the TPM treatment group (*51*).

*Levetiracetam (Keppra®) (**17**)*. Levetiracetam (LEV), a water soluble pyrrolidone derivative, is the S-enantiomer of α-ethyl-2-oxo-pyrrolidine acetamide. Although LEV shares some targets (such as delayed rectifier channels and N- and P/Q-type calcium channels) with other AEDs, it is a novel AED with a unique mechanism of action related to an interaction with synaptic vesicle protein 2A (SV2A) (*52*). This anticonvulsant drug is structurally related to the nootropic drug piracetam. In contrast to the activity of the (*S*)-isomer, the (*R*)-form of LEV was at least 150-fold less potent in the audiogenic seizure susceptible mouse and largely inactive in other models (*53*). It has been approved by the USFDA for adjunctive therapy in the treatment of partial-onset seizures in patients 16 years of age and older with epilepsy (*54*).

LEV analog. Seleteracetam and brivaracetam exemplifiethe analog of prototype anticonvulsant LEV that exerts site selectivity and illustrates the possibility of widening the target specificity respectively.

*Seletracetam (**18**)*. Seletracetam (STM) (UCB 44212; (2*S*)-2-[(4*S*)-4-(2,2-difluorovinyl)-2-oxopyrrolidin-1-yl] butanamide, is a structural analog of the AED levetiracetam which binds selectively and stereospecifically to SV2A (a novel binding site, synaptic vesicle protein 2A) with a 10-fold greater affinity than LEV. SV2A is thought to be involved with synaptic vesicle exocytosis and neurotransmitter release (*55*). SV2A represents a novel molecular target that seems to have an important role in the pharmacological activity of STM. The SV2A protein is thought to assist with the coordination of synaptic vesicle exocytosis and neurotransmitter release (*56*). Proteins involved in exocytosis, and SV2 in particular, could be considered as promising novel targets for the development of new CNS drug therapies. Discovering the mechanism of drug action through this receptor triggered a drug discovery program which led to the identification of brivaracetam (currently in phase III clinical trials for epilepsy), and seletracetam. Studies show that STM binds selectively to SV2A, without direct modulation of Na^+ channels (*57,58*). A study was performed by Klitguard and coworkers on an *in vitro* high K^+ low Ca^{2+} concentration fluid (HKLCF) model of epilepsy (mice and rat) and they have concluded that STM induces a more potent and complete suppression of neuronal synchronization than LEV. Furthermore, STM showed no psychomimetic effects and a very high tolerability index in both kindled and GAERS rats, which is markedly superior to that of LEV and other AEDs (*59*). Apart from various similarities seletracetam differs from levetiracetam by a very potent and selective effect against Zn^{2+} inhibition of glycine-gated currents as well as a more potent inhibition of high-voltage-operated Ca^{2+} currents and epileptiform elevation of intracellular Ca^{2+} concentrations involving multiple high-voltage-operated Ca^{2+} channels. In pharmacokinetic studies seletracetam was found to reach C_{max} within 1 h. The linear, time-independent pharmacokinetics of the drug combined with a rapid and almost complete absorption indicates that STM has a major uncomplicated pharmacokinetic profile (*60*).

*Brivaracetam (UCB 34714) (**19**)*. has had a drug discovery program carried out by Kenda *et al*. (2004) (*61*) for ligands with significant affinity to LBS (levetiracetam binding site) as a novel molecular target. Brivaracetam (BVT) ((2*S*)-2-[(4*R*)-2-oxo-4-propylpyrrolidinyl] butanamide), the 4-*n*-propyl structural analog of LEV, emerged as the single most

potent drug candidate among a series of compounds having a 4-substituted lactam ring by small-sized hydrophobic groups. It is approximately 10 times more potent than LEV as an antiseizure agent in audiogenic seizure-prone mice. Further Tai and Truong (2007) (*62*) demonstrated that BVT possesses more potent anti-seizure and anti-myoclonic activity than LEV in an established rat model of cardiac arrest induced post-hypoxic myoclonus. It possesses a binding affinity for the synaptic vesicle protein 2A (SV2A) and also shows an ability to inhibit Na^+ channels. BVT has a half-life of 8 h and its metabolites are not pharmacologically active. In various experimental models of epilepsy, brivaracetam exhibited properties superior to LEV as an AED and has an excellent tolerability profile in humans (*63*). A phase II clinical trial established that it produced a dose-dependent reduction in the frequency of seizures in adults with refractory partial seizures. The drug is currently under phase III clinical trials (*64*).

*Zonisamide (Zonegran®) (**20**).* Zonisamide (ZNS) is a benzisoxazole with a sulfonamide side chain (1, 2-benzisoxazole-3-methanesulfonamide). The main actions of ZNS are blockade of sodium channels and reduction of voltage dependent T-type Ca^{2+} currents (*65*). It also enhances neuronal inhibition *via* modulation of neurotransmitter systems, including dopaminergic, GABAergic and serotonergic systems. It may enhance GABA function through interaction at allosteric or other binding sites and/or by influencing GABA transport. ZNS is also reported to be a weak inhibitor of carbonic anhydrase (*66*). It has a favorable pharmacokinetic profile as it is rapidly and completely absorbed and has a long half-life (63-69 h in healthy volunteers) which allows twice-daily, or even once-daily dosing. ZNS undergoes acetylation to form *N*-acetyl ZNS, and reduction to form the open ring metabolite, 2-sulfamoylacetyl phenol (**21**) that undergoes urinary excretion *via* glucuronide conjugation (*67*). It has been shown to be effective in patients whose seizures are resistant to other AEDs. Patients most often received ZNS as monotherapy. When ZNS was added to therapy with other AEDs, the dosage of the other AEDs was reduced. ZNZ or CBZ are favored over phenytoin, clobazam, valproate, or phenobarbital for simple partial and complex partial seizures (*68*). The drug has been approved in the US and Europe as an adjunctive therapy for refractory partial seizures in adults. It has several CNS dose-dependent side effects and by slow titration of dose the incidence could be minimized (*69*). A recent observational study was carried out by Kelemen *et al*. for accessing the efficacy and tolerability of ZNS in different resistant generalized epileptic syndromes. At a mean dose of 367 mg/day (100-600 mg/day), it was observed that ZNS showed excellent efficacy against progressive myoclonic epilepsy type 1. They suggested that the free radical scavenging and possible neuroprotective effects of zonisamide may be beneficial in acquired symptomatic epilepsies (*70*).

*Lacosamide (Vimpat®) (**22**).* Lacosamide (LCM) (formally known as harkoseride) is a new AED discovered by high throughput animal screening. Systematic evaluation of more than 100 *N*-benzyl-2-acetamidopropionamide derivatives of this compound in animal models led to the identification of LCM (*71*). It is the first drug to come from a class of compounds known as functionalized amino acids and it is an optical antipode of the naturally occurring amino acid L-serine (*72*). It has dual mode of action as LCM enhances slow inactivation of voltage-gated sodium channels and modulates the collapsin response mediator protein-2 (CRMP-2), a protein, which is part of neuronal signal transduction pathways and which is attributed to neuroprotection (*73*). In contrast to AEDs such as phenytoin, CBZ, and LTG that block sodium channels when activated, LCM facilitates slow inactivation of sodium channels both in terms of kinetics and voltage dependency (*74*). Clinically, LCM is at present in a late stage of development as an adjunctive treatment for patients with uncontrolled partial-onset seizures. It provides high oral bioavailability unaffected by food, good tolerability with twice daily dosing, and minimal drug-drug interactions (*75*).

*Rufinamide (Banzel®; Inovelon®) (**23**).* Rufinamide (RFM) (1-[(2,6-difluorophenyl) methyl]-1*H*-1,2, 3-triazole-4-carboxamide) is a triazole derivative structurally unrelated to any currently marketed AEDs. Comparative studies of rufinamide with established AEDs (phenytoin, phenobarbital, ethosuximide, valproate) in several rodent seizure models showed the superiority of rufinamide to other AEDs tested in terms of protective indices in the electrically and chemically induced seizure tests, and the MES safety ratio (*76*). The drug is effective orally and is relatively well absorbed in the lower dose range. The main route of metabolism involves hydrolysis of the carboxamide group by carboxylesterases to an inactive derivative that is eliminated mainly by renal excretion *via* glucuronide conjugation (*77*). The precise mechanisms of action of RFM are unknown, however *in vitro* studies suggest that modulation of sodium channels activity, particularly prolongation of the inactive state may be the main mechanism of its antiepileptic activity. It possesses several favourable properties which might pave its way as the orphan drug for the treatment of partial seizures and drop attacks associated with Lennox-Gastaut syndrome (*78*). Coppola *et al.* (2011) (*79*) showed that it is also effective and well tolerated as an adjunctive drug for the treatment of refractory childhood-onset epileptic encephalopathies. Vendrame *et al*. (2010) (*80*) in single-centric studies observed that it is also has potential for treatment of a wide range of other seizure types including both partial and generalized epilepsy syndromes in the pediatric population. Further it showed no effect on cognitive function in patients with refractory partial seizures (*81*).

*DP-valproic acid (DP-VPA) (**24**).* DP-VPA (SPD 421, DP 16, TVA, RAP-valproate), a novel prodrug of VPA (**25**) in which the VPA moiety is covalently bound to the phospholipid lecithin based on a new drug delivery technology known as Regulated Activation of Prodrug (D-RAPTM). The ED_{50} value is 50-fold lower than VPA with a longer half-life. It showed a high absorption rate and bioavailability with negligible hepatic metabolism. Side effects are restricted to dose-dependent gastrointestinal problems (*82*). It is currently under development for the treatment of partial and generalized seizures (*83*). The absorption pattern of DP-VPA follows a unique pattern whereby the complex permeates through the gut wall and enters intact to the enterocyte. Then it associates itself with chylomicrons and reaches the systemic blood circulation *via* the lymphatic route (*84*).

*Eslicarbazepine acetate (BIA 2-093) (**26**).* Eslicarbazepine acetate (ESL) [(*S*)-(−)-10-acetoxy-10,11-dihydro-5*H*-dibenz[b,f]azepine-5-carboxamide], formerly known as BIA 2-093 is a novel central nervous system (CNS)-active agent. It belongs to the members of first-line AEDs represented by carbamazepine (first-generation) and oxcarbazepine (second-generation) having the dibenz/b,f/azepine nucleus bearing the 5-carboxamide substitute but is structurally different at the 10,11-position (*85*). This molecular variation results in differences in metabolism, preventing the formation of toxic epoxide metabolites such as carbamazepine-10,11 epoxide. It is the prodrug of ESL (*S*-licarbazepine (**27**)), the entity responsible for pharmacological activity. It is currently under clinical development for the treatment of epilepsy and bipolar disorder and acts by inhibiting voltage-gated sodium channels. Among the other dibenz[b,f] azepine-5-carboxamide derivatives ESL has the highest protective index (*86*). A recent phase III study of ESL demonstrated that ESL in a once-daily dosage of 800 and 1,200 mg was effective in reducing standardized seizure frequency. It was well tolerated as adjunctive therapy for partial-onset seizures in patients who were refractory to treatment with standard AED therapy. Mild to moderate dizziness, headache, diplopia, somnolence, and vertigo were the most commonly reported dose related adverse effects (*87*).

*Ganaxolone (**28**).* Ganaxolone (GNX) (3α-hydroxy-3β-methyl-5α-pregnan-20-one), a neuroactive steroid currently in clinical trials represents a potential AED. It is a beta methylated synthetic analogue of allopregnanolone (3α-hydroxy-21xi, 22-oxido-21-homo-5α-pregnan-20-one) and thought to act through positive allosteric modulation of the $GABA_A$ receptor (*88*). In healthy human volunteers, GNX, administered in doses ranging from 50 to 1,500 mg, either as drug alone or formulated with pharmaceutical grade excipients, is rapidly absorbed from the gastrointestinal tract after oral administration. The 3β-methyl substituent minimizes metabolism at the 3β-hydroxyl group so GNX is orally active, is not converted to the hormonally active 3-keto form, and hence lacks hormonal side effects (*89*). GNX has been shown to be well tolerated in adults and children and the commonly observed adverse events in children were agitation and somnolence. It is currently undergoing further development against newly diagnosed infantile spasms (*90*), in adults with refractory partial-onset seizures (*91*) and in women with catamenial epilepsy (*92*).

*Losigamone (**29**).* Losigamone (LSG), a racemic mixture of 5-α-5 (2-chlorphenylhydroxmethyl)-4-methoxy-2(5*H*)-furanone is related to β-methoxy-butenolides and is very similar to fadyenolides and piperolides isolated from *Piper fadyenii* and *Piper sanctum*, respectively. It is the first drug that has been identified using medicinal plant-based drug discovery (*93*). The mechanism of action for LSG is not clearly known, although several have been proposed (*94*). Initially it was thought that the anticonvulsant effect of losigamone may be due to NMDA (*N*-methyl-D-aspartic acid) antagonism and inhibition of excitatory amino acid release (*95*). *In vitro* and *in vivo* experiments carried out on genetically epilepsy prone DBA/2 mice by Jones and Davies suggested that the clinically effective anticonvulsant activity of LSG is attributed to its *S* (+)-enantiomer rather than *R* (−)-enantiomer or its racemic mixture (*96*). Present data suggests that the drug decreases neuronal excitability *via* a decrease in the persistent Na^+ current in rat hippocampal neurons (*97*). In a multi-center, double-blind, randomized clinical trial LSG was found to be an effective and safe add-on drug for refractory partial epilepsy in adults. The median reduction in seizure frequency as well as responder rate was significantly greater for a dose of 1,500 mg/day than for 1,200 mg/day, indicating a dose-response relationship (*98*).

*Remacemide hydrochloride (**30**).* Remacemide hydrochloride (RMD) [(±)-2-amino-*N*-(1-methyl-1,2-diphenylethyl)-acetamide monohydrate] is a racemic mixture and the (–) isomer is more potent than the (+) isomer in a maximal electroshock seizure (MES) test in mice. The major route of metabolism of remacemide involves desglycination and the principal metabolite is desglycinyl-remacemide (DGR). The drug may be considered as a prodrug because the anticonvulsant effects of the drug may be primarily mediated by DGR (*99*). It is a two-fold more potent Na^+ channel blocker and a 100-fold more potent non-competitive NMDA channel antagonist. Further, it also exhibits a greater efficacy than RMD itself in a variety of animal seizure models (*100*). Regardless of the activity of DGR, RMD exhibits inconsistent clinical efficacy as add-on therapy because DGR appears to be more susceptible to hepatic enzyme induction than the parent compound (*101*). Studies also showed that remacemide was significantly less effective than carbamazepine in preventing seizure

recurrence. Although significant pharmacodynamic interactions were observed between remacemide and other AEDs (valproate, CBZ, phenytoin, and phenobarbital) (*102*), unfavorable pharmacokinetic interactions make RMD an unsuitable candidate for adjunctive treatment of epilepsy (*103*).

*Retigabine (**31**)*. Retigabine (RTG), *N*-(2-amino-4-(4-fluorobenzylamino) phenyl) carbamic acid ethyl ester effective against partial-onset seizures is the first novel KCNQ opener in the late stages of clinical development with an excellent safety profile (*104*). Rundfeldt (1997) (*105*) demonstrated that RTG initiates a membrane conductance which is selective for K^+ ions and it contributes to the anticonvulsant activity. Further studies established that it acts as a KCNQ potassium channel opener that involves opening of neuronal Kv7.2 (KCNQ2) voltage activated K^+ channels (*106*). Besides opening of peripheral KCNQ channels it hyperpolarizes the axotomized terminals that may constitute a novel and selective mechanism for attenuation of neuropathic pain symptoms (*107*). RTG as an adjunctive drug displayed promising improvement in patients with partial drug-resistant epilepsy. The most prominent adverse effects due to retigabine add-on therapy were dizziness, somnolence, and fatigue. It is metabolized primarily by glucuronidation to *N*-glucuronide metabolites and by acetylation (*108*).

*Safinamide (**32**)*. By retaining the acetamide portion and replacement of the pentylamino moiety of milacemide (with residues present in the structures of substrates and inhibitors of the MAO (Mono Amine Oxidase), Pevarello *et al*. (1998) (*109*) derived the lead 2-[[4-(3-chlorobenzoxy)benzyl]amino]acetamide. As an outcome of this study, safinamide (SAF), ((*S*)-2-[[4-(3-fluorobenzoxy)benzyl]amino]propanamide methanesulfonate) (**33**), a 2-substituted amino amide emerged as a potent, orally active AED with a good safety margin. It has been shown to antagonize the calcium and sodium channels; as well as inhibit monoamine oxidase type-B (MAO-B) and the inhibition is reversible. Selectivity of SAF for the B isoform of the enzyme versus A is 5,000 and 1,000 times higher in rat and human brains, respectively (*110*).

*Soretolide (D-2916) (**34**)*. Soretolide (SRT), (2,6-dimethylbenzamide *N*-(5-methyl-3-isoxazolyl)) is a new potent anticonvulsant exhibiting similar pharmacological properties to those of carbamazepine. Maurizis *et al*. hypothesized that SRT follows two metabolic degradation pathways. The active metabolite D3187 (**35**) has a better ability to cross the blood-brain barrier than the unchanged drug in female rats which may be attributed to the longer anticonvulsant activity of SRT (*111*). It is effective in the MES test in rodents but STR and its active metabolite are ineffective in protecting against PTZ-induced clonic seizures and in blocking generalized seizures in the hippocampal kindling rat model (*112*).

*Stiripentol (**36**)*. Stiripentol (STP) is an efficient drug for add-on therapy in severe myoclonic epilepsy in infancy. When combined with CBZ and clobazam, it prevents the formation of the inactive metabolite of CBZ, epoxy-carbamazepine, and hydroxylation of the active metabolite of clobazam into hydroxy-norclobazam respectively (*113*). *In vitro* and *in vivo* studies suggest that STP can be considered as a "booster" of clobazam as the inhibitory effect of STP on CYP2C19 (Ki = 0.14 μM) was found to potentiate the antiepileptic effect of clobazam (*114*). It is positive allosteric modulator acting directly upon the $GABA_A$ receptor. Although it does not solely depend on the subunit composition of the receptor, STP elicits higher activity at the α3- or δ-subunit containing receptors. This drug with target selectivity is of particular importance if the said receptor subunits are responsible for any kind of neuronal dysfunction associated with neuronal hyperexcitability (*115*).

*AMPA receptor antagonist talampanel (**37**)*. 2-Amino -3-(3-hydroxy-5-methyl-4-isoxazolyl) propionic acid receptors (AMPARs) play important roles in neurotransmission in the CNS and in the synaptic plasticity that underlies learning processes and memory (*116*). However, under certain pathological conditions the AMPARs over-activation determines neuronal cell death related to various neurological diseases such as stroke, Huntington's chorea, epilepsy, *etc*. Therefore, AMPAR antagonists have been considered useful as therapeutic agents for these disorders, particularly in epileptic seizures and are emerging as a promising new target for epilepsy therapy (*117*). The majority of the researches on AMPAR receptor antagonists are on the non-competitive (allosteric) AMPAR antagonists interacting with an allosteric AMPA binding site (Figure 3). The non-competitive antagonists have the advantage of remaining effective independently of the level of glutamate or the polarization state of the synaptic membrane during a neurological disease (*118*).

Moreover, they do not influence the normal glutamatergic activity after prolonged use. Thus, in recent years some important classes of these ligands have been developed. The first lead to be identified as a selective, non-competitive AMPA receptor antagonist is 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466) (**38**) (*119*). Based on this template, various 2,3-benzodiazepine compounds were synthesized and evaluated. Among all the compounds, the dioxolo-benzodiazepine talampanel also named LY300164 emerged as a highly active molecule. Its phase II clinical trials in the U.S. in patients with severe epilepsy not responsive to other drugs have yielded positive results. Phase III trials in epilepsy are underway to confirm and expand these results (*120*). Talampanel (TLP) (GYKI-53773, LY300164), a non-competitive AMPA receptor blocker has undergone initial assessment in patients with

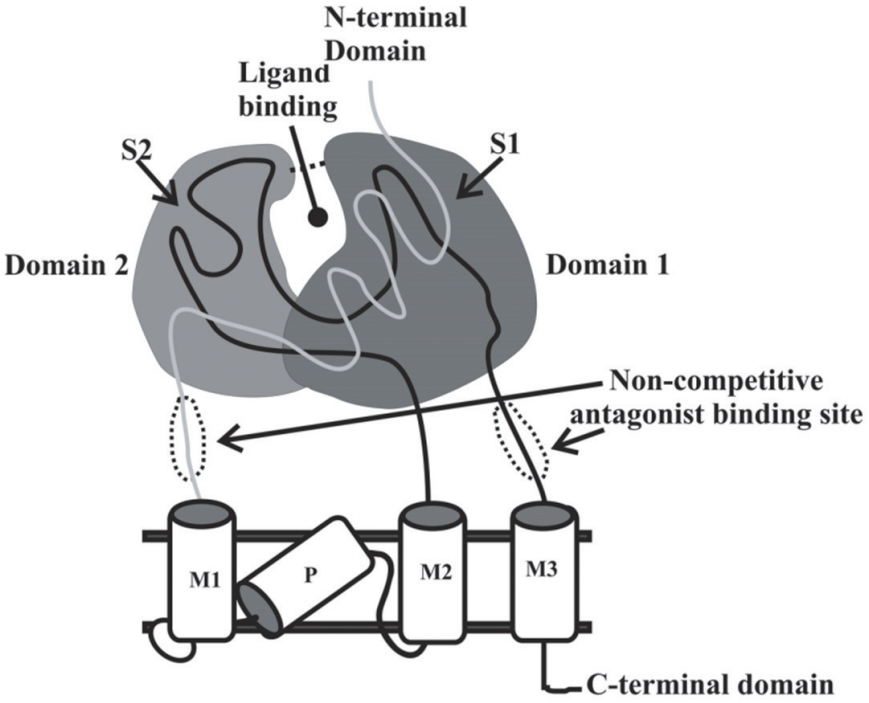

Figure 3. Schematic model of an AMPA receptor subunit. The model illustrates the agonist binding core (such as glutamate and AMPA) and competitive antagonist binding site. The putative sites of action of allosteric non-competitive antagonist talampanel are also shown. The agonist binding core consists of two domains (S1 and S2) attached by linkers to the cation channel domain consisting of three membrane-spanning segments (M1-M3) and a pore loop (P). The C-terminal domain is intracellular and the *N*-terminal domain is extracellular. (Figure reproduced with permission, Bialer *et al*., 2007)

epilepsy. In an early adjunctive-therapy crossover trial in 49 patients with refractory partial seizures, median seizure frequency was 21% lower on talampanel than on the placebo, a statistically significant effect (*121*). A recent review that deals with talampanel extensively summarizes that the antiepileptic is generally well tolerated in adults with refractory complex partial seizures. The most commonly reported adverse event is dizziness and at higher doses sedation and ataxia may occur (*122*). A new potent noncompetitive AMPA receptor antagonist perampanel (**39**) that has demonstrated efficacy and good tolerability in the treatment of refractory partial onset seizures is in late stage clinical development (*123*). Whether the drug will prove adequately effective to reduce the morbidity and mortality of epilepsy is yet to be ensured.

*Valrocemide/ TV1901 (**40**)*. Valrocemide (VGD) is a combination of VPA and glycinamide (*N*-valproyl glycinamide), a chemical derived from glycine, an amino acid that can have an antiepileptic effect if its concentration in the brain is increased (*124*). Isoherranen *et al*. (2001) (*125*) investigated the anticonvulsant activity of VGD in various animal (rodent) models of human epilepsy to determine its anticonvulsant profile and safety margin. The results obtained in this study suggest that VGD has a broad spectrum of anticonvulsant activity and promising potential as a new AED. VGD is currently under development by Teva and Acorda therapeutics as a potential therapeutic for the treatment of epilepsy. In the year 2003, a phase II trial using valrocemide as an adjunct therapy in refractory epilepsy patients had been completed and phase III trials were being planned. VGD was also being investigated for potential utility in the treatment of bipolar disorder and neuropathic pain (*126*).

*Pregabalin (Lyrica®) (**41**)*. Pregabalin (PGB) is the alkylated analogue of the neurotransmitter GABA. Its binds potently to the α2γ subunit, an auxiliary protein associated with voltage-gated calcium channels (VGCC) in the CNS and reduces calcium influx at nerve terminals thereby (*127*) modulating the release of excitatory neurotransmitters in "hyper-excited" neurons, restoring them to normal physiological state (*128*). It is indicated as an adjunctive therapy in adults with partial seizures with or without secondary generalization, peripheral neuropathic pain and in patients with generalized anxiety disorder or social anxiety disorder (*129*). A recent study by Briggs *et al*. suggested that PGB at a higher dose is effective in reducing the absolute frequency of secondarily generalized tonic-clonic seizures in patients with clinically refractory partial epilepsy, but not secondary generalization (*130*).

The majority of the newer AEDs used clinically are derived from structural modification of the existing drugs. These include vigabatrin, oxcarbazepine, fluorofelbamate, brivaracetam, DP-valproic acid, eslicarbazepine, valrocemide, and pregabalin. The drugs are developed with an objective to augment the efficacy and safety margin and few of them are

effective in combination. Although it may provide a solution to contained epilepsy an absolute seizure-free state is still not attainable and often "evergreening" may be one of the criteria for such structural modifications. This phenomenon of existing drug modification also does not encourage sustained research for exploring new targets. Nevertheless, few drugs acting on newer molecular targets *viz.*, SV2A protein, AMPA receptor *etc*. with promising clinical trial results might shape a better therapeutic outcome. Few currently available investigational agents act at diverse targets that are involved in the pathogenesis of this complex neurological disorder and therefore, it is impossible to anticipate all the agents that provide an equivalent level of potency and efficacy. Additionally, the complexity increases when taking into consideration the nature of epileptogenesis in an individual patient. Application of plant-based drug discovery is still in its infancy as far as epilepsy is concerned and demands more screening of this novel source for generation of leads or prototype drugs as exemplified by losigamone.

3. Pharmacoresistance

Pharmacoresistance to medication is an important clinical problem in epilepsy. The phenomenon is observed in approximately one third of patients, and is associated with an increased risk of death and other ill consequences. Pharmacoresistance in epilepsy may be defined as seizures that continue to occur despite treatment trials with at least three appropriate AEDs at maximum tolerated doses (*131*). When a patient fails to respond to two or three appropriate AEDs then the chance of significant benefit from other drugs is 10% or less (*132*).

There are a number of factors associated with drug resistance and varies from one patient to another including early age of seizure onset combined with high seizure frequency, type of epileptic syndrome and seizure, structural brain lesions (*e.g.* cortical dysplasia) or electroencephalographic abnormalities and history of status epilepticus (*133*). At present two hypotheses have been asserted to explain the development of pharmacoresistance to AEDs *viz.*, the target hypothesis and the transporter hypothesis. The target hypothesis denotes that epilepsy related changes in the molecular properties of the drug targets contribute to pharmacoresistance. For example, Remy *et al*. (2003) (*134*) studied cellular mechanisms underlying drug resistance in resected hippocampal tissues from patients. They suggested that a loss of Na^+ channel drug sensitivity may constitute the development of resistance. The transporter hypothesis accentuates that resistance develops due to overexpression or increase in functioning of multidrug transporters in the brain, leading to poor penetration of AEDs into brain targets and thereby contributing to multidrug resistance (MDR) in epilepsy. To substantiate the hypothesis, Volk and Loscher using a rat model of temporal lobe epilepsy demonstrated that there is an increase in expression of multidrug transporter proteins such as the ATP-binding cassette sub-family B member 1 (ABCB1, also known as MDR1 and P-glycoprotein 170) in the brain of the rat with drug-resistant spontaneous seizures (*135*). Siddiqui *et al*. (2003) (*136*) recognized a genetic factor associated with resistance to AEDs. They hypothesized that polymorphisms in the drug transporter gene (CC genotype at the *ABCB1* C3435T) is associated with increased expression of the ABCB1 protein which inturn influences the response to AED treatment. Although no genetic stratification is underpinned, a recent study found a significant association between *ABCB1* polymorphisms and drug resistance when patients were stratified by the same type of epilepsy and/or in those treated with the same AEDs (*137*). Researchers' efforts might target development of AEDs that are not recognized by MDR proteins or that can evade ABCB1. Alternatively, agents that inhibit these proteins (*138*) can be concomitantly administered with the currently available AEDs thereby decreasing the incidence of pharmacoresistance.

4. Combination therapy

Monotherapy is generally recommended for patients with newly diagnosed epilepsy. Combination therapy (CT) should only be initiated upon unresponsiveness to monotherapy. As the mechanism of action does not generally provide much guidance while combining AEDs, it is important to know the efficacy of each drug in different seizure types. CT has been found to be successful in about 30% of patients. A non-randomized trial suggested the efficacy of combination therapy in achieving seizure-freedom in epilepsies refractory to single drug treatment (*139*). Drug interaction is a common phenomenon observed during CT. This may be avoided by choosing non-interacting drugs. If such alternatives are not available, interacting drugs may be administered together by monitoring the plasma drug concentration followed by adjustment of dosage (*140*). For attaining a viable therapeutic outcome, CT requires rational combination to be tailored on an individual basis. In general, if the efficacy of two AEDs combined is shown to be additive or supra-additive and the burden of side effects is less than additive, the combination is considered to be advantageous. On the contrary if there is no observed efficacy or it is less than additive while the side effect burden is equal to additive, the combination is regarded as unfavorable (*141*). With reservation to evidence-based findings, Karceski *et al*. (2009) (*142*) summarized the choice of specific medications as possible add-on agents that were identified as showing efficacy based on Class I and Class II evidence in the 2004 American Academy

Table 2. Combination/add-on therapy

Existing agent	Appropriate add-ons	
	Symptomatic localization related epilepsy	Idiopathic generalized epilepsy
Felbamate	–	***Valproate***, lamotrigine
Lamotrigine	***Levetiracetam***, topiramate, oxcarbazepine	Valproate, topiramate, levetiracetam, zonisamide
Levetiracetam	***Lamotrigine***, ***carbamazepine***, oxcarbazepine	***Valproate***, lamotrigine, topiramate
Topiramate	***Lamotrigine***, ***levetiracetam***, carbamazepine	***Lamotrigine***, ***valproate***, levetiracetam
Valproate	***Levetiracetam***, oxcarbazepine, lamotrigine	***Lamotrigine***, topiramate, levetiracetam, zonisamide
Vagus nerve stimulation	–	***Valproate***, lamotrigine, topiramate
Zonisamide	Levetiracetam, lamotrigine	***Lamotrigine***, ***valproate***, levetiracetam
Tiagabine	***Lamotrigine***, levetiracetam, topiramate	–
Phenytoin	***Levetiracetam***, ***lamotrigine***	–
Phenobarbital	***Lamotrigine***, ***levetiracetam***, oxcarbazepine	–
Oxcarbazepine	***Levetiracetam***, lamotrigine, topiramate	–
Gabapentin	***Lamotrigine***, ***levetiracetam***, oxcarbazepine	–
Carbamazepine	***Levetiracetam***, lamotrigine	–

Bold italic indicates treatment of choice.

of Neurology (AAN)/American Epilepsy Society (AES) guidelines (Table 2). Among the various possible combinations, the information from the table implied that LEV may be considered as the universal add-on drug. Furthermore, recent evidence revealed that a combination of LEV with other AEDs, particularly those enhancing GABAergic inhibition, lead to additive/synergistic effects on seizure protection with minimal side effects and pharmacokinetic interactions (*143*).

5. Conclusion

Despite the discovery of a number of AEDs, the management of epilepsy still remains an intricate task. Due to the prevalence of resistance to monotherapy, combination therapy proves workable. The utilization of available drugs to combat resistance requires rational adaptation of data arising out of clinical trials. Most of the currently available AEDs possess multiple mechanisms of action. With a few exceptions, the precise primary mode of action of some newer AEDs remains to be discovered. A multidiciplinary approach to identify potential receptor site, mechanism of action, and reason for resistance would pave the way for better therapeutic interventions towards the management of epilepsy.

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Brief Report

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Anti-inflammatory activities of fractions from *Geranium nepalense* and related polyphenols

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ABSTRACT: *Geranium nepalense* Sweet is a common Chinese herbal medicine and has been used as influenza, dysentery, antiphlogistic and analgesic tonic, hemostatic, stomachic, and antidiabetic drugs. The anti-inflammatory effects of *G. nepalense* on tetradecanoyl phorbol acetate (TPA)-induced mouse ear edema were studied in this work. The results showed that ethyl acetate fraction of the water extract of *G. nepalense* possessed significant activity at 2.5 g/kg ($p < 0.01$) with aspirin as a positive control (0.6 g/kg). Six polyphenolic compounds, including three flavonoids, *i.e.* kaempferol, kaempferol-7-*O*-β-D-glucopyranoside, and quercetin-7-*O*-α-rhamnopyranoside, and two tannins, *i.e.* pyrogallol and gallic acid, and one lignin, *i.e.* epipinoresinol, were isolated and characterized from ethyl acetate fraction. The isolation of polyphenols provides a clue for beneficial effects of *G. nepalense* in the demonstrated anti-inflammatory activity.

Keywords: *Geranium nepalense*, anti-inflammatory activity, TPA-induced mouse ear edema, polyphenolic, flavonoids

1. Introduction

Geranium nepalense Sweet (Geraniaceae) is widely distributed in China (*1*). It has been used to treat various inflammatory conditions, including influenze, dysentery, antiphlogistic and analgesic, and used as a Chinese herbal medicine (*2,3*). The extract of *G. nepalense* inhibited tetradecanoyl phorbol acetate (TPA)-induced edema in mouse ears in our screening for anti-inflammatory components. Here, we report the anti-inflammatory activities of extract fractions and the compounds isolated from the active extract of *G. nepalense*.

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2. Materials and Methods

2.1. *General experimental procedures*

The NMR spectra were obtained on a Bruker AM-400 spectrometer operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR, respectively. The spectra of electro spray ionization-mass spectrometry (ESI-MS) were recorded on a Finnigan LCQ Advantage Max ion trap mass spectrometer (Thermo Finnigan, USA). The isolation process was conducted on silica gel (200-300 meshes, Qingdao Marine Chemical, China), Sephadex LH-20 (25-100 μm, Fluka, Switzerland). Thin layer chromatography (TLC) was carried out on silica gel GF254 plates (0.2 mm thickness, 5 × 10 cm, Qingdao Marine Chemical, China).

2.2. *Plant material*

G. nepalense Sweet was collected in Songhuaba, Kunming, Yunnan, China. The authentication process was carried out by Dr. Jianying Xiang (Kunming Institute of Botany, Chinese Academic of Sciences). A voucher specimen was deposited in the Kunming Institute of Botany, Chinese Academic of Sciences (Kunming, Yunnan, China).

2.3. *Animals*

The Kunming mice were purchased from Yunnan Baiyao Group Company Limited (Kunming, Yunnan, China). Animal Ethics Committee (AEC) approvals were obtained for the experimental protocols. The AEC oversees animal programs, facilities and procedures. Mice were housed in a climate-controlled environment with a 12 h light/dark cycle and were provided with free access to food and water during the experiment.

2.4. *Extraction and fractionation*

The dried and cut material (2.0 kg) was soaked in distilled water and boiled for three times. The water solution was combined and concentrated *in vacuo* to about 500 mL. The concentrated water solution was then partitioned with ethyl acetate and *n*-butanol successively. After revaporation under reduced pressure, ethyl acetate fraction (37 g) (marked as GN-EA) and *n*-butanol fraction (33 g) (marked as GN-BU) were obtained respectively. Finally, the left water fraction was concentrated to dry and marked as GN-W.

2.5. *Anti-inflammatory activities of extract fractions of G. nepalense*

The anti-inflammatory activities of GN-EA, GN-Bu, and GN-W were evaluated in a TPA-induced mouse ear edema model. The inflammation model was established according to Hu's and Agut's method (*4,5*).

A total of 75 Kunming mice were allotted to five groups of 15 each in a completely randomized design. The mice of each group were treated by gastric perfusion of none, 0.6 g/kg aspirin, 2.5 g/kg GN-EA, 2.5 g/kg GN-Bu, and 7.5 g/kg GN-W 30 min prior to each TPA (0.05 mL) treatment once a day for 3 days.

The mice were sacrificed 1 h after the last TPA treatment. Ear punches (7 mm diameter) were taken from each group and weighted. The *t* test with different samples was adopted for comparison between groups.

2.6. *Isolation of GN-EA fraction*

The ethyl acetate fraction (32 g) was subjected to column chromatography (CC) over silica gel eluted with a solvent system of $CHCl_3$/MeOH in gradient (100:1; 100:5; 100:10, and MeOH) to obtain 9 subfractions (Fr. 1-9) based on the TLC analysis. Fr. 4 (606 mg) was subjected to CC over silica gel (15 g) and eluted with petroleum ether/acetone (10:1; 5:1; 3:1) to obtain Fr. 4a (25 mg) and further purified by CC over Sephadex LH-20 eluted with MeOH to yield **1** (8 mg). Fr. 8 was purified by repeated CC over Sephadex LH-20 eluted with MeOH to yield **2** (90.5 mg) and **3** (24.0 mg). Fr. 5 (900 mg) was subjected to CC over Sephadex LH-20 eluted with MeOH to obtained **4** (670 mg). Fr. 6 was purified by CC over Sephadex LH-20 eluted with MeOH to yield **5** (700 mg). Fr. 1 (400 mg) was subjected to CC over silica gel (15 g) and eluted with petroleum ether/acetone (10:1; 5:1) to obtain Fr. 1a (25 mg). Fr. 1a was further purified by CC over Sephadex LH-20 eluted with MeOH to yield **6** (6.5 mg).

3. Results and Discussion

3.1. *Anti-inflammatory activities of G. nepalense fractions*

Preliminary phytochemical screening and evaluation of anti-inflammatory components indicated that organic acids, flavonoids, polyphenolic, tannin, and essential oil may be responsible, at least in part, for the anti-inflammatory effects of the total extract of *Geranium* (*3,6*). Therefore, the TPA-induced ear edema model was employed with the objective of seeking the major bioactive fraction from *G. nepalense*. Both GN-EA and GN-Bu fractions exhibited significant ($p < 0.01$) anti-inflammatory activities on TPA-induced ear edema model at 2.5 g/kg in our study (Table 1).

3.2. *Chemical structures of the isolated compounds*

The ethyl acetate extract of *G. nepalense* was isolated by repeated column chromatography (Sephadex LH-20 and silica gel) to afford six pure compounds. These compounds were subjected to ^{1}H-NMR, ^{13}C-NMR, and ESI-MS analyses for structure identification (Data are shown in the Appendix). They are elucidated to be kaempferol (**1**) (*7-9*), kaempferol-7-*O*-β-D-glucopyranoside (**2**) (*10-12*), quercetin-7-*O*-α-rhamnopyranoside (**3**) (*13*), pyrogallol (**4**) (*14*), gallic acid (**5**) (*15*), and epipinoresinol (**6**) (*16*) according to their ^{1}H- and ^{13}C-NMR spectral data and compared with spectral values in literatures. This is the first report for the isolation of compounds **1**-**6** (Figure 1) from this plant.

Table 1. Anti-inflammatory activity of GN fractions of *G. nepalense*

Treatment	Rats (individuals)	Dose (g/kg)	Weight of ear edema (mg)
Model	15	0.0	7.33 ± 3.04
Aspirin	15	0.6	2.84 ± 1.94**
GN-EA	15	2.5	2.48 ± 2.02**
GN-Bu	15	2.5	3.36 ± 1.76**
GN-W	15	7.5	5.62 ± 2.86#

All values are expressed as mean of 15 mice in each group. Statistically significant: ** $p < 0.01$ compared to control, # compared to GN-EA.

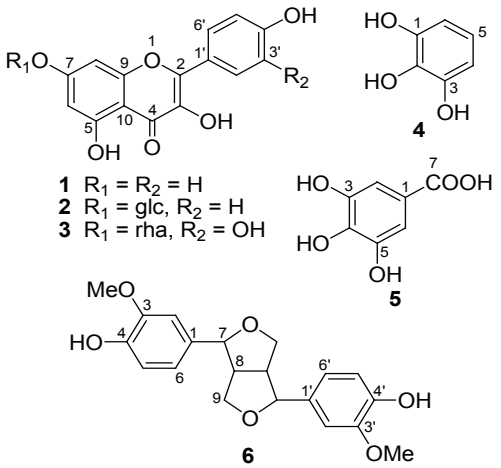

Figure 1. The chemical structures of compounds 1-6.

Previously studies have suggested that the ethyl acetate extract of *G. carolinianum* was the anti-inflammatory active fraction (*17*). The polyphenolic compounds of *Geranium* spp. such as flavonoids and tannins have been shown to possess free radical scavenging/antioxidant anti-inflammatory activity both *in vivo* and *in vitro* (*12,18,19,20*). The anti-inflammatory effect of *G. nepalense* was evaluated by TPA-induced ear edema model *in vivo* in this study. The results showed that both GN-EA and GN-Bu fractions inhibited TPA-induced inflammation. Six compounds were isolated from the GN-EA fraction. The chemical structures of them were elucidated to be flavonoids (**1-3**), pyrogallol (**4**), gallic acid (**5**), and epipinoresinol (**6**), which all belong to polyphenolic. **1** isolated from *Hibiscus cannabinus* L. showed significant anti-inflammatory (*21*). **3** and **6** were also isolated from the leaves of *Brasenia schreberi*, and both compounds exhibited anti-inflammatory activities (*22*). Our results suggested that highly-enriched **4** and **5** may be the best active constituents related to the traditional utilization of this herb.

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Appendix

^{1}H-NMR, ^{13}C-NMR, and MS analyses of the isolated compounds 1-6.

*Kaempferol **(1)***, yellow powder, $C_{15}H_{10}O_6$, ESI-MS: *m/z* 285 $[M - H]^-$. ^{1}H-NMR (400 Hz, DMSO-d_6) δ: 7.98 (d, J = 8.8, H-2′, 6′, 2H), 6.96 (d, J = 8.8, H-3′, 5′, 2H), 6.74 (d, J = 2.0, H-8), 6.36 (d, J = 2.0, H-6). ^{13}C-NMR (100 Hz, DMSO-d_6) δ: 150.3 (C-2), 138.7 (C-3), 178.4 (C-4), 161.4 (C-5), 92.3 (C-6), 160.2 (C-7), 97.7 (C-8), 160.9

(C-9), 105.2 (C-10), 130.5 (C-1′), 130.2 (C-2′, 6′), 115.6 (C-3′, 5′), 155.3 (C-4′).

Kaempferol-7-O-β-D-glucopyranoside ***(2)***, $C_{21}H_{20}O_{11}$, ESI-MS: *m/z* 447 $[M - H]^-$. ^{1}H-NMR (400 Hz, CD_3OD) δ: 8.25 (d, *J* = 8.8, H-2′, 6′, 2H), 7.73 (d, *J* = 8.8, H-3′, 5′, 2H), 6.25 (d, *J* = 2.1, H-8), 6.19 (d, *J* = 2.1, H-6), 5.82 (d, *J* = 9.7, glc-H-1). ^{13}C-NMR (100 Hz, CD_3OD) δ: 146.8 (C-2), 138.7 (C-3), 176.4 (C-4), 161.4 (C-5), 98.7 (C-6), 164.2 (C-7), 93.7 (C-8), 157.1 (C-9), 104.6 (C-10), 132.0 (C-1′), 128.6 (C-2′, 6′), 115.4 (C-3′, 5′), 155.6 (C-4′), 103.8 (glc-C-1), 75.0 (glc-C-2), 78.6 (glc-C-3), 71.3 (glc-C-4), 79.0 (glc-C-5), 62.4 (glc-C-6).

Quercetin-7-O-α-rhamnopyranoside ***(3)***, $C_{21}H_{20}O_{11}$, ESI-MS: *m/z* 447 $[M - H]^-$; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.33 (d, *J* = 1.5 Hz, H-2'), 6.90 (d, *J* = 8.3 Hz, H-5'), 7.29 (dd, *J* = 1.5, 8.3 Hz, H-6'), 6.19 (br. s, H-6), 6.35 (br. s, H-8), 5.34 (br. s, rha-H-1), 0.93 (d, *J* = 6.1 Hz, rha-H-6); ^{13}C-NMR (100 MHz, CD_3OD) δ: 148.9 (C-2), 136.2 (C-3), 179.6 (C-4), 158.5 (C-5), 99.8 (C-6), 165.8 (C-7), 94.7 (C-8), 158.2 (C-9), 105.9 (C-10), 133.0 (C-1'), 116.4 (C-2'), 146.4 (C-3',), 147.8 (C-4'), 117.0 (C-5'), 123.9 (C-6'), 103.5 (rha-C-1), 71.9 (rha-C-2), 72.0 (rha-C-3), 72.1 (rha-C-4), 73.3 (rha-C-5), 17.6 (rha-C-6).

Pyrogallol ***(4)***, brown needle, $C_6H_6O_3$, EI-MS *m/z* (%): 126 (100, M^+); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 6.79 (t, *J* = 8.0, H-5), 6.63 (d, *J* = 8.0, H-4, 6, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 110.7 (C-4, C-6), 120.7 (C-5), 140.0 (C-2), 147.8 (C-1, 3).

Gallic acid ***(5)***, colorless needles, $C_7H_6O_5$, EI-MS *m/z* (%): 170 (100, M^+), 153 (80), 126 (92); ^{1}H-NMR (400 MHz, C_5D_5N) δ: 8.08 (s, H-2, H-6); ^{13}C-NMR (100 MHz, C_5D_5N) δ: 123.0 (C-1), 110.7 (C-2, C-6), 147.8 (C-3, C-5), 140.7 (C-4), 169.9 (C-7).

Epipinoresinol ***(6)***, white powder, $C_{20}H_{22}O_6$, ESI-MS: *m/z* 357 $[M - H]^-$; ^{1}H-NMR (400 MHz, C_5D_5N) δ: 4.94 (d, *J* = 3.3, H-7, 7', 2H), 3.23 (m, H-8, 8', 2H), 4.31 (m, H-9a, 9'a, 2H), 4.00 (m, H-9b, 9'b, 2H), 3.76 (s, C-3, 3'-OMe, 6H); ^{13}C-NMR (100 MHz, C_5D_5N) δ: 133.2 (C-1, 1'), 111.0 (C-2, 2'), 148.9 (C-3, 3'), 147.9 (C-4, 4'), 116.5 (C-5, 5'), 119.8 (C-6, 6'), 86.5 (C-7, 7'), 54.8 (C-8, 8'), 72.0 (C-9, 9'), 58.2 (C-3, 3'-OMe).

Original Article

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Synthesis and structure-activity relationship study of 2-(substituted benzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one derivatives as anticancer agents

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ABSTRACT: The synthesis and structure-activity relationship (SAR) study of a series of 2-(substituted benzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one (4a-4j) derivatives as anticancer agents are described. This series of thiazolopyrimidines were synthesized by the reaction of 7-(4-fluoro phenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*] pyrimidin-3(7*H*)-one (3) with appropriate substituted aldehydes in the presence of anhydrous sodium acetate and glacial acetic acid. Their structures were confirmed by IR, ^{1}H-NMR, mass, and elemental analyses. These novel thiazolopyrimidine derivatives were screened for their anticancer activity on the U937 human histocytic lymphoma cell line by 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. The comparison of anticancer activity of thiazolopyrimidine was performed considering their structures. This study was done using 2-(substituted benzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one (4a-4j) as a basic model, showing that *i*) presence of a hydrogen donor/ acceptor domain [thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one] on the thiazolopyrimidine ring; *ii*) presence of a hydrophobic [(4-fluorophenyl)] aryl ring system on the thiazolopyrimidine ring; *iii*) presence of an electron donor moiety [5-(furan-2-yl)] on the thiazolopyrimidine ring; *iv*) *ortho* and *para* substitution of the distal aryl ring [2-(substituted benzylidene)] function strongly influenced anticancer activity. Among these compounds (4a-4j) *para* substituted derivatives 4c, 4e, 4f, 4g, 4h, and 4j showed significant anticancer activity.

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1. Introduction

Thiazole, pyrimidine and related pyrimidines are classes of fused heterocycles that are of considerable interest because of the diverse range of their biological properties. These are among a wide variety of nitrogen heterocycles that have been explored for developing pharmaceutically important molecules. Thiazolopyrimidine and related fused heterocycles are of interest as potential bioactive molecules, which can be considered as thia-analogues of the natural purine bases such as adenine and guanine, and have acquired a growing importance in the field of medicinal chemistry because of their biological potential. They are known to exhibit pharmacological activities such as analgesic, antiinflammatory, antiarrhythmic, antiparkinsonian, and anticancer activities (*1-8*).

Cancer is a collection of different life threatening diseases characterized by uncontrolled growth of cells leading to invasion of surrounding tissue and often spreading to other parts of the body. When it comes to understanding and controlling cancer scientists are now working from a position of strength because a foundation of knowledge about cancer has been built over the past 50 years. There is an urgent need for novel effective drug regimens for the treatment of cancer because the current chemotherapy suffers from a slim therapeutic index, with significant toxicity from effective drug doses or tumor recurrence at low drug doses. The new anticancer chemotherapeutic agents search continues to be an active area of research at many companies and research centers (*9*,*10*). Searching for new anticancer agents having heterocyclic nucleus continues worldwide at various laboratories (*11-13*).

In the last several decades, fused pyrimidine

derivatives are a class of heterocyclic compounds that have attracted significant interest in medicinal chemistry because they have a wide range of pharmaceutical and pharmacological applications including potential anti-tumor, antimycobacterial, and antiviral activities. Moreover, in recent years, it was reported that many fused pyrimidine analogues were reported to be inhibitors of tyrosine kinase and cyclin-dependent kinases, which are involved in mediating the transmission of mitogenic signals and numerous other cellular events (*14-19*), including, cell proliferation, migration, differentiation, metabolism, and immune responses. It was also found that many of these derivatives may block proliferation of various cancer cell lines (*20*).

Led by the above facts on pyrimidine chemistry, we have synthesized new 2-(substituted benzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one derivatives (**4a-4j**). The main objective of the present investigation is a modified experimental approach to evaluate position of substitutions in synthesized compounds and validation with reference drugs such as gefitinib. The introduction of the substituted benzylidene at the 2nd position of the thiazolopyrimidine scaffold led to significant anticancer activity.

2. Materials and Methods

2.1. *Chemicals and reagents*

The chemicals and reagents used were obtained from various chemical units including Aldrich Co. (Powai, Mumbai, India), E. Merck India Ltd. (Ponda, Goa, India), CDH (Daryaganj, New Delhi, India), and SD Fine Chem (Worli Road, Mumbai, India). These solvents used were of laboratory research (LR) grade and purified before their use. The silica gel G used for analytical chromatography (TLC) was obtained from E. Merck India Ltd. Melting points were measured in open capillary tubes on a Boetius apparatus (Carl Zeiss Jena) and are uncorrected. ^{1}H-NMR spectra were taken on a 300 MHz NMR spectrometer (model Ultra Shield, Bruker, Rheinstetten, Germany) in (d_6-DMSO) using tetramethylsilane [$(CH_3)_4Si$] as internal standard. Chemical shifts (δ) are expressed in ppm. Mass spectra were obtained on an instrument (JEOL-SX-102, Japan) using electron impact ionization. IR spectra were recorded in KBr pellets on a Fourier-transform infrared spectrometer (FT-IR 410, Jasco Corporation, Tokyo, Japan). Elemental analyses were performed on an elemental analyzer (Model 240c, Perkin Elmer, Thane, Maharashtra, India) and were within ± 0.4% of the theoretical values.

2.2. *General procedure for the synthesis of title compounds (**4a-4j**)*

2.2.1. *Preparation of 3-(4-fluorophenyl)-1-(furan-2-yl)prop-2-en-1-one (**1**)*

The key intermediates were synthesized by a previously reported method (*21*). 3-(4-Fluorophenyl)-1-(furan-2-yl) prop-2-en-1-one (**1**) prepared by the mixture of KOH (0.055 mol), water (20 mL), ethanol (15 mL), 2-acetyl furan (0.043 mol), and *p*-fluorobenzaldehyde (0.043 mol) was stirred at 30-40°C for 2 h and kept overnight. It was then filtered, washed with water and with ethanol, dried and refluxed with glacial acetic acid (10 mL) for 2 h. The crystals separated after cooling were filtered and washed with water, dried and used in further reactions. Yield 79%, Mp 212°C; IR (KBr) cm^{-1}: 2,991 (Ar-CH_{str}), 1,733 (C=O), 1,631 (C=C), 1,030 (cyclic C–O–C_{str}), 823 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δppm): 7.51 (d, J = 8.2 Hz, 2H, ArH), 7.23 (dd, J_1 = 7.7 Hz, J_2 = 1.83 Hz, 2H, ArH), 6.53-7.21 (m, 3H, –CH-furan), 6.10-7.14 (d, 2H, =CH); MS (EI) m/z 216 [M]$^+$; Anal. Calcd. for $C_{13}H_9FO_2$: C, 72.22; H, 4.20; Found: C, 72.23; H, 4.22.

2.2.2. *4-(4-Fluorophenyl)-6-(furan-2-yl)-3,4-dihydropyrimidin-2(1H)-thione (**2**)*

A mixture of 3-(4-fluorophenyl)-1-(furan-2-yl)prop-2-en-1-one (**1**) (0.039 mol) thiourea (0.03 mol) and potassium hydroxide (2.5 g) in 95% ethanol (100 mL) was heated under reflux for 3 h. The reaction mixture was concentrated to half of its volume, diluted with water, then acidified with dilute acetic acid and kept overnight. The solid thus obtained, was filtered, washed with water and recrystallized from ethanol to give 4-(4-fluorophenyl)-6-(furan-2-yl)-3,4-dihydropyrimidine-2(1*H*)-thione (**2**). Yield 72%, Mp 231°C; IR (KBr) cm^{-1}: 3,361 (NH_{str}), 3,021 (Ar-CH_{str}), 1,531 (C=C), 1,034 (cyclic C–O–C_{str}), 843 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.39 (dd, J_1 = 6.4 Hz, J_2 = 1.8 Hz, 2H, ArH), 7.41 (d, J = 8.2 Hz, 2H, ArH), 6.61-6.81 (m, 3H, –CH-furan), 6.14 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 1H, pyrimidine H), 4.70 (dd, J_1 = 12.0 Hz, J_2 = 2.0 Hz, 1H, pyrimidine H), 3.27 (s, 2H, –NH); MS (EI) m/z 274 [M]$^+$; Anal. Calcd. for $C_{14}H_{11}FN_2OS$: C, 61.30; H, 4.04; N, 10.21; Found: C, 61.33; H, 4.06; N, 10.24.

2.2.3. *7-(4-Fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**3**)*

The chloroacetic acid (0.096 mol) was melted on a water bath and (**2**) (0.009 mol) added to it portion wise to maintain its homogeneity. The homogeneous mixture was further heated on a water bath for 30 min and kept overnight. The solid thus obtained was washed with water and recrystallized from ethanol to give 7-(4-fluorophenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one (**3**). Yield 69%, Mp 227°C; IR (KBr) cm^{-1}: 3,354 (NH_{str}), 3,047 (Ar-CH_{str}), 1,721 (C=O), 1,512 (C=C), 1,021 (cyclic C–O–C_{str}), 822 (C-Cl); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.31 (dd, J_1 = 7.1 Hz, J_2 = 2.2 Hz, 2H, ArH), 7.74 (d, J = 7.9 Hz, 2H, ArH), 6.71-7.12 (m, 3H, –CH-furan), 5.32 (dd, J_1 = 7.5 Hz, J_2 = 2.1 Hz, 1H, thiazolopyrimidine H), 4.13 (dd, J_1 = 12.1 Hz, J_2 = 1.8 Hz, 1H, thiazolopyrimidine H), 3.61 (s,

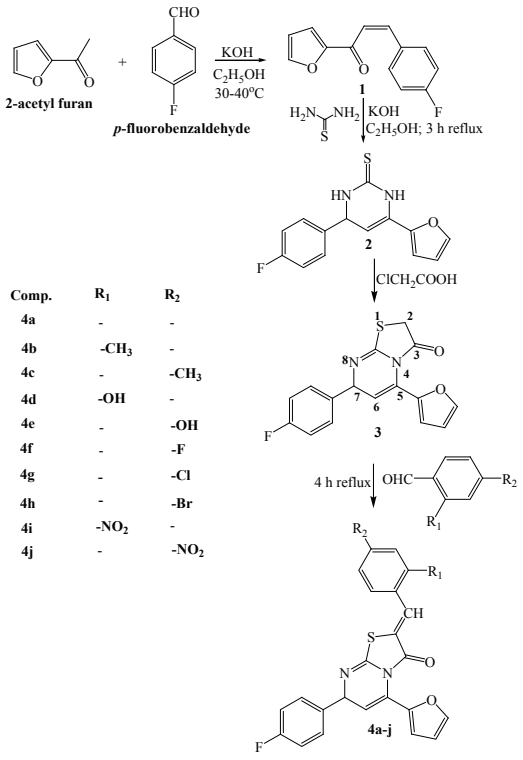
2H, –CH_2 thiazole); MS (EI) m/z 314 $[M]^+$; Anal. Calcd. for $C_{16}H_{11}FN_2O_2S$: C, 61.14; H, 3.53; N, 8.91; Found: C, 61.12; H, 3.51; N, 8.94.

2.2.4. *General procedure for the synthesis of 2-(substitutedbenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4a-4j**)*

A mixture of (**3**) (0.002 mol), substituted benzaldehyde (0.002 mol), and anhydrous sodium acetate (0.002 mol) in 100% glacial acetic acid (10 mL) was heated under reflux for 4 h. The reaction mixture was kept overnight and the solid, thus separated, was filtered, washed with water and recrystallized from ethanol to furnish 2-(substituted benzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one (**4a-4j**) (Scheme 1).

2.3. *Cell proliferation assay*

The U937 human histocytic lymphoma cell line was obtained from cell line bank of National Center for Cellular Sciences (NCCS), Pune, India. These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C, in a CO_2 incubator in the presence or absence of test compounds. The anticancer property of the compounds was measured by MTT assay (*22*). The cells were plated in a 96-well plate at a density of 5,000 cells/well. After 24 h, cell culture media was replaced with DMEM containing 10% FBS and the cells were treated with different concentrations of the compounds



Comp.	R_1	R_2
4a	-	-
4b	$-CH_3$	-
4c	-	$-CH_3$
4d	-OH	-
4e	-	-OH
4f	-	-F
4g	-	-Cl
4h	-	-Br
4i	$-NO_2$	-
4j	-	$-NO_2$

Scheme 1. Synthetic protocols of target compounds (4a-4j).

(0.01-50 mM). The cells were later incubated for 72 h. Cytotoxicity was measured by adding 5 mg/mL of MTT to each well and incubating for another 3 h. The purple formazan crystals were dissolved by adding 100 µL of DMSO to each well. The absorbance was read at 570 nm in a spectrophotometer. Cell death was calculated as follows: Cell death percentage = 100 – [test absorbance/ control absorbance] × 100. The test result is expressed as the concentration of a test compound which inhibits the cell growth by 50% (IC_{50}).

3. Results and Discussion

3.1. *Chemistry*

The chemical structures of the synthesized compounds were confirmed by infrared spectroscopy, proton nuclear magnetic resonance spectroscopy, mass spectrometry, and elemental analysis. The presence of the carbonyl and olefinic group in compound (**1**) is characterized by the presence of two strong bands in its IR spectrum at 1,733 and 1,631 cm^{-1}. The formations of compound (**2**) were confirmed by NH stretching, bending peaks in the range of 3,382, 1,621 cm^{-1}, and appearance of a singlet peak 3.27 for two protons in its ^{1}H-NMR spectra which might be assigned to NH group connecting the pyrimidine. The conversion of thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one (**3**) can be recognized by a strong absorption peak at 1,721cm^{-1} in IR due to the carbonyl group in the thiazole ring. The title compounds (**4a-4j**) showed a singlet at δ 7.12, 7.35, 7.32, 7.31, 7.14, 7.22, 7.25, 7.23, 7.19, and 7.32 ppm due to the benzylidine ring proton in ^{1}H-NMR confirms the formation of (**4a-4j**) respectively. Further mass spectra confirmed their purity and molecular weight.

3.2. *Biological activity*

All the selected compounds 2-(substituted benzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*] pyrimidin-3(7*H*)-one (**4a-4j**) were evaluated for cytotoxic properties on the U937 human histocytic lymphoma cell line with gefitinib as a standard positive control. Inhibition of cell-proliferation was measured by MTT assay. The inhibitory potency (IC_{50}) of compounds (**4a-4j**) are given in Table 1. The fact that a majority of clinically active anticancer drugs possess a nitrogen hetero atomic system with one or two phenyl rings, at least one carbonyl group in their structure and the presence of hydrogen donor/ acceptor unit is noted. In all of the pioneering experiments important core fragments (*23*) are defined by the presence of a hydrogen donor/acceptor unit (HAD), a hydrophobic domain (A) (aryl ring substituted/unsubstituted) and an electron donor atom (D). These common features were found in the structures of well-established anticancer drugs such as gefitinib, erlotinib, lapatinib, and dasatinib as well as synthesized compounds (Figure 1). In general,

Table 1. Anticancer study of synthesized compounds (4a-4j) on U937 human histocytic lymphoma cell line

Compounds	R_1	R_2	IC_{50} (μM) ± SEM[a]
4a	-	-	10.04 ± 0.52
4b	$-CH_3$	-	12.07 ± 0.31
4c	-	$-CH_3$	5.37 ± 0.12
4d	-OH	-	15.12 ± 0.22
4e	-	-OH	5.81 ± 0.17
4f	-	-F	3.04 ± 0.26
4g	-	-Cl	4.04 ± 0.32
4h	-	-Br	3.51 ± 0.43
4i	$-NO_2$	-	20.12 ± 0.26
4j	-	$-NO_2$	6.42 ± 0.21
Gefitinib	-	-	1.00 ± 1.00

[a] Mean of three independent experiments ± mean standard error.

lipophilicity is one of the most important parameters because it is mainly involved in pharmacokinetic processes such as absorption, distribution, metabolism, excretion, and toxicity (ADMET) and in ligand-target interactions (*24*). Lipophilicity is the molecular parameter of choice in numerous quantitative structure-activity relationships (QSAR) of different classes of compounds (*25*). The promising activity of the compounds may be attributed to the substitutions on the hydrophobic domain. These compounds contain methyl, hydroxy, nitro, and halogens at the *para* position of the benzylidene aryl ring. Moreover, observed data showed that the *para* substituted derivatives exhibited better activity than other *ortho* and unsubstituted derivatives. Compounds **4f** and **4h** were found to be most potent and showed IC_{50} values of 3.04 and 3.51 mM, respectively. When the *ortho* group was on the benzylidene aryl ring of compounds, we found a decrease in inhibitory activity. For example, compounds **4d** and **4i** were 3-fold less potent than **4e** and **4j**. Among the ten compounds synthesized, we found that compounds **4f** and **4h** showed comparable activity to that of gefitinib. Moreover, *p*-CH_3, *p*-OH, and *p*-NO_2 substituted compounds (**4c**, **4e**, and **4j**) had slighter lower activity than (**4f-4h**). However, the unsubstituted and *ortho* substituted (*o*-methyl, *o*-hydroxyl, and *o*-nitro) compounds (**4a**, **4b**, **4d**, and **4i**) exhibited lesser activity. The anticancer activity of test compounds with decreasing order is shown in Figure 2 and is tabulated in Table 1.

3.3. *Structure activity relationships (SAR) study*

SAR studies give insights into molecular properties causing receptor affinity and selectivity. The promising nature of the compounds may be attributed to the substitutions on the hydrophobic domain (benzylidene aryl ring). These compounds had electron withdrawing and donating groups at the *ortho* and *para* positions of the hydrophobic aryl ring. In general it was observed that the *para* substituted derivatives were more active than the other derivatives. This may be because of the fact that the *para* substituted derivatives fit better into the receptor site.

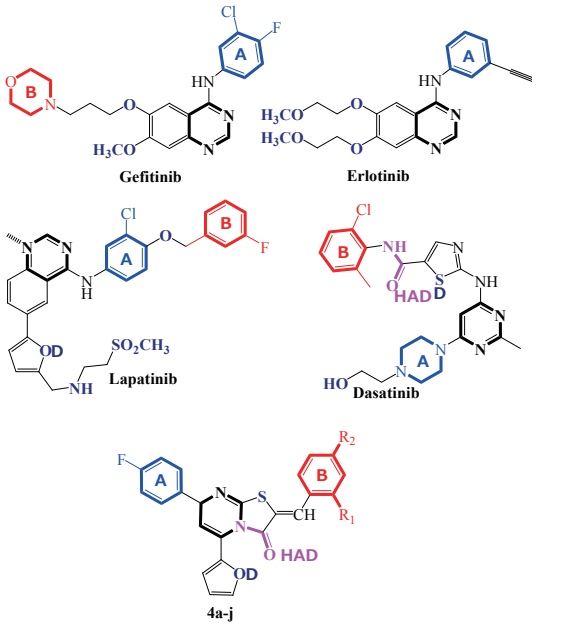


Figure 1. Vital core fragments of wellknown anticancer drugs and synthesized compounds with its important structural features: (HAD) hydrogen bond acceptor/donor domain. (A) hydrophobic aryl ring system, **(B)** distal aryl ring system, and **(D)** electron donor moiety.

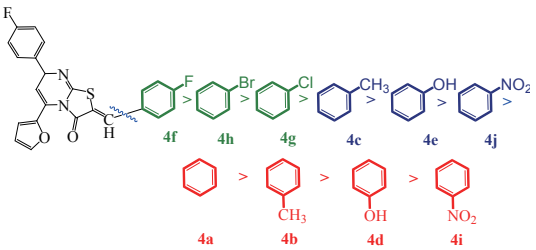


Figure 2. Decreasing order of anticancer activity of thiazolopyrimidine derivatives.

Based on these general concepts we planned to prepare different modifications in title compounds, namely, *ortho* and *para* substitution in the thiazolopyrimidine ring. These modifications showed insight into the dependency of the receptor binding efficacy. Furthermore the *para* substituted thiazolopyrimidine skeletal structure conserved the good receptor binding results. Finally *para* substituted compounds **4c**, **4e**, **4f**, **4g**, **4h**, and **4j** exhibited significant anticancer activity.

4. Conclusions

The literature survey revealed that *para* substitution on the phenyl ring appeared to greatly influence pharmacological activity. This research examined anticancer properties of a novel series of 2-(substituted benzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2*H*-thiazolo[3,2-*a*]pyrimidin-3(7*H*)-one (**4a-4j**) compounds. Results revealed that para substituted derivatives exhibited better anticancer activity.

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Appendix

Spectral data of the synthesized compounds

*2-Benzylidene-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4a**)*

Yield 73%, Mp 284°C; IR (KBr) cm^{-1}: 3,061 (Ar-CH_{str}), 1,642 (C=O), 1,514 (C=C benzylidine), 1,032 (cyclic C–O–C_{str}), 811 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.41 (dd, J_1 = 7.3 Hz, J_2 = 1.8 Hz 2H, ArH), 7.53 (d, J = 6.2 Hz, 2H, ArH), 7.56-7.87 (m, 5H, Ar-H), 7.12 (s, 1H, –CH benzylidine), 6.51-6.89 (m, 3H, –CH-furan), 5.16 (dd, J_1 = 8.1 Hz, J_2 = 2.2 Hz 1H, thiazolopyrimidine H), 3.21 (dd, J_1 = 17.6 Hz, J_2 = 1.8 Hz 1H, thiazolopyrimidine H); MS (EI) m/z 402 $[M]^+$; Anal. Calcd. for $C_{23}H_{15}FN_2O_2S$: C, 68.64; H, 3.76; N, 6.96; Found: C, 68.69; H, 3.73; N, 6.92.

*2-(2-Methylbenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4b**)*

Yield 76%, Mp 291°C; IR (KBr) cm^{-1}: 3,047 (Ar-CH_{str}), 1,623 (C=O), 1,521 (C=C), 1,031 (cyclic C–O–C_{str}), 837 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.24 (dd, J_1 = 6.4 Hz, J_2 = 1.4 Hz 2H, ArH), 7.24 (d, J = 8.2 Hz, 2H, ArH), 7.31-7.54 (m, 4H, Ar-H), 7.35 (s, 1H, –CH benzylidine), 6.14-6.66 (m, 3H, –CH-furan), 5.43 (dd, J_1 = 8.6 Hz, J_2 = 2.9 Hz 1H, thiazolopyrimidine H), 3.24 (dd, J_1 = 11.6 Hz, J_2 = 1.6 Hz 1H, thiazolopyrimidine H), 2.71 (m, 3H, –CH_3); MS (EI) m/z 416 $[M]^+$; Anal. Calcd. for $C_{24}H_{17}FN_2O_2S$: C, 69.21; H, 4.11; N, 6.73; Found: C, 69.24; H, 4.13; N, 6.71.

*2-(4-Methylbenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4c**)*

Yield 82%, Mp 297°C; IR (KBr) cm^{-1}: 3,057 (Ar-CH_{str}), 1,632 (C=O), 1,543 (C=C), 1,031 (cyclic C–O–C_{str}), 811 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.33 (dd, J_1 = 7.2 Hz, J_2 = 1.8 Hz, 2H, ArH), 7.54 (d, J = 7.7 Hz, 2H, ArH), 7.67-7.71 (m, 4H, Ar-H), 7.32 (s, 1H, –CH benzylidine), 6.23-6.74 (m, 3H, –CH-furan), 5.43 (dd, J_1 = 8.1 Hz, J_2 = 1.7 Hz, 1H, thiazolopyrimidine H), 3.14 (dd, J_1 = 13.2 Hz, J_2 = 2.2 Hz, 1H, thiazolopyrimidine H), 2.25 (m, 3H, –CH_3); MS (EI) m/z 416 $[M]^+$; Anal. Calcd. for $C_{24}H_{17}FN_2O_2S$: C, 69.21; H, 4.11; N, 6.73; Found: C, 69.18; H, 4.14; N, 6.75.

*2-(2-Hydroxybenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4d**)*

Yield 82%, Mp 276°C; IR (KBr) cm^{-1}: 3,412 (phenolic OH), 3,043 (Ar-CH_{str}), 1,643 (C=O), 1,512 (C=C), 1,021 (cyclic C–O–C_{str}), 819 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 9.93 (s, 1H; Ar-OH), 7.36 (dd, J_1 = 7.6 Hz, J_2 = 2.0 Hz, 2H, ArH), 7.51 (d, J = 7.5 Hz, 2H, ArH), 7.63-7.74 (m, 4H, Ar-H), 7.31 (s, 1H, –CH benzylidine) 6.23-6.65 (m, 3H, –CH-furan), 5.41 (dd, J_1 = 8.7 Hz, J_2 = 1.8 Hz, 1H, thiazolopyrimidine H), 3.13 (dd, J_1 = 12.4 Hz, J_2 = 1.9 Hz, 1H, thiazolopyrimidine H); MS (EI) m/z 418 $[M]^+$; Anal. Calcd. for $C_{23}H_{15}FN_2O_3S$: C, 66.02; H, 3.61; N, 6.69; Found: C, 66.06; H, 3.64; N, 6.67.

*2-(4-Hydroxybenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4e**)*

Yield 79%, Mp 294°C; IR (KBr) cm^{-1}: 3,471 (phenolic OH), 3,037 (Ar-CH_{str}), 1,624 (C=O), 1,511 (C=C), 1,037 (cyclic C–O–C_{str}), 817 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 9.71 (s, 1H, Ar-OH), 7.16 (dd, J_1 = 7.2 Hz, J_2 = 2.2 Hz, 2H, ArH), 7.26 (d, J = 7.1 Hz, 2H, ArH), 7.35-7.48 (m, 4H, Ar-H), 7.14 (s, 1H, –CH benzylidine), 6.26-6.59 (m, 3H, –CH-furan), 5.36 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, thiazolopyrimidine H), 3.17 (dd, J_1 = 11.8 Hz, J_2 = 2.0 Hz, 1H, thiazolopyrimidine H); MS (EI) m/z 418 $[M]^+$; Anal. Calcd. for $C_{23}H_{15}FN_2O_3S$: C, 66.02; H, 3.61; N, 6.69; Found: C, 66.06; H, 3.64; N, 6.71.

*2-(4-Fluorobenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4f**)*

Yield 73%, Mp 289°C; IR (KBr) cm^{-1}: 3,055 (Ar-CH_{str}), 1,636 (C=O), 1,536 (C=C), 1,035 (cyclic C–O–C_{str}), 822 (C-F), 912 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.47 (dd, J_1 = 6.8 Hz, J_2 = 1.4 Hz 2H, ArH), 7.53 (d, J = 7.0 Hz, 2H, ArH), 7.62-7.96 (m, 4H, Ar-H), 7.22 (s, 1H, –CH benzylidine), 6.21-6.76 (m, 3H, –CH-furan), 5.42 (dd, J_1 = 7.6 Hz, J_2 = 1.2 Hz, 1H, thiazolopyrimidine H), 3.19 (dd, J_1 = 12.5 Hz, J_2 = 2.4 Hz, 1H, thiazolopyrimidine H); MS (EI) m/z 420 $[M]^+$; Anal. Calcd. for $C_{23}H_{14}F_2N_2O_2S$: C, 65.71; H, 3.36; N, 6.66; Found: C, 65.74; H, 3.37; N, 6.68.

*2-(4-Chlorobenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4g**)*

Yield 75%, Mp 291°C; IR (KBr) cm^{-1}: 3,053 (Ar-CH_{str}), 1,634 (C=O), 1,532 (C=C), 1,031 (cyclic C–O–C_{str}), 834 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.41 (dd, J_1 = 7.1 Hz, J_2 = 1.8 Hz, 2H, ArH), 7.56 (d, J = 6.8 Hz, 2H, ArH), 7.61-7.96 (m, 4H, Ar-H), 7.25 (s, 1H, –CH benzylidine), 6.22-6.77 (m, 3H, –CH-furan), 5.42 (dd, J_1 = 7.9 Hz, J_2 = 1.8 Hz, 1H, thiazolopyrimidine H), 3.11 (dd, J_1 = 14.2 Hz, J_2 = 2.0 Hz, 1H, thiazolopyrimidine H); MS (EI) m/z 438 [M+2]; Anal. Calcd. for $C_{23}H_{14}ClFN_2O_2S$: C, 63.23; H, 3.23; N, 6.41; Found: C, 63.26; H, 3.22; N, 6.44.

*2-(4-Bromobenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4h**)*

Yield 72%, Mp 286°C; IR (KBr) cm^{-1}: 3,051 (Ar-CH_{str}), 1,643 (C=O), 1,544 (C=C), 1,041 (cyclic C–

O–C_{str}), 851 (C-F), 621 (C-Br); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.41 (dd, J_1 = 7.9 Hz, J_2 = 2.0 Hz, 2H, ArH), 7.53 (d, J = 6.3 Hz, 2H, ArH), 7.61-7.94 (m, 4H, Ar-H), 7.23 (s, 1H, –CH benzylidine), 6.26-6.75 (m, 3H, –CH-furan), 5.43 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, thiazolopyrimidine H), 3.12 (dd, J_1 = 16.1 Hz, J_2 = 2.2 Hz, 1H, thiazolopyrimidine H); MS (EI) m/z 483 [M+2]; Anal. Calcd. for $C_{23}H_{14}BrFN_2O_2S$: C, 57.39; H, 2.93; N, 5.82; Found: C, 57.35; H, 2.91; N, 5.86.

*2-(2-Nitrobenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4i**)*

Yield 79%, Mp 293°C; IR (KBr) cm^{-1}: 3,054 (Ar-CH_{str}), 1,647 (C=O), 1,549 (C=C), 1,047 (cyclic C–O–C_{str}), 842 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.86 (dd, J_1 = 8.4 Hz, J_2 = 1.8 Hz, 2H, ArH), 7.91 (d, J = 6.9 Hz, 2H, ArH), 8.02-8.35 (m, 4H, Ar-H), 7.19 (s, 1H, –CH benzylidine), 6.32-6.59 (m, 3H, –CH-furan), 5.36 (dd, J_1 = 8.0 Hz, J_2 = 1.8 Hz, 1H, thiazolopyrimidine H), 3.12 (dd, J_1 = 14.2 Hz, J_2 = 2.0 Hz, 1H, thiazolopyrimidine H); MS (EI) m/z 447 $[M]^+$; Anal. Calcd. for $C_{23}H_{14}FN_3O_4S$: C, 61.74; H, 3.15; N, 9.39; Found: C, 61.77; H, 3.11; N, 9.37.

*2-(4-Nitrobenzylidene)-7-(4-fluorophenyl)-5-(furan-2-yl)-2H-thiazolo[3,2-a]pyrimidin-3(7H)-one (**4j**)*

Yield 81%, Mp 286°C; IR (KBr) cm^{-1}: 3,051 (Ar-CH_{str}), 1,643 (C=O), 1,547 (C=C), 1,041 (cyclic C–O–C_{str}), 887 (C-F); ^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 7.81 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 2H, ArH), 7.94 (d, J = 6.3 Hz, 2H, ArH), 8.12-8.37 (m, 4H, Ar-H), 7.32 (s, 1H, –CH benzylidine), 6.19-6.52 (m, 3H, –CH-furan), 5.47 (dd, J_1 = 8.2 Hz, J_2 = 2.2 Hz, 1H, thiazolopyrimidine H), 3.14 (dd, J_1 = 12.4 Hz, J_2 = 1.8 Hz, 1H, thiazolopyrimidine H); MS (EI) m/z 447 $[M]^+$; Anal. Calcd. for $C_{23}H_{14}FN_3O_4S$: C, 61.74; H, 3.15; N, 9.39; Found: C, 61.71; H, 3.17; N, 9.35.

Original Article

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Antioxidant and hepatoprotective activity of an ethanol extract of *Syzygium jambos* (L.) leaves

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ABSTRACT: Herbal medicines have traditionally been used worldwide for the prevention and treatment of liver disease with fewer adverse effects. The leaves of the *Syzygium jambos* (SJL) plant were chosen and studied for their antioxidant activity *in vitro* and hepatoprotective activity *in vivo*. The antioxidant activity of the ethanol extract was examined *in vitro* using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, reducing capacity, total phenol, total flavonoid content, and total antioxidant capacity. The extract had significant dose-dependent antioxidant activity in all *in vitro* experiments. IC_{50} values of SJL and ascorbic acid (standard) were found to be 14.10 and 4.87 µg/mL, respectively, according to a DPPH radical scavenging assay. Hepatoprotective activity of the plant extract was evaluated in a rat model of carbon tetrachloride (CCl_4)-induced liver damage. CCl_4 significantly altered serum marker enzymes, total bilirubin, total protein, and liver weight. The extract caused these values to return to normal in rats with CCl_4-induced liver damage that were given SJL. This indicated the hepatoprotective potential of SJL and was comparable to use of the standard drug silymarin. Thus, the present study revealed that SJL may have antioxidant and hepatoprotective activity.

Keywords: *Syzygium jambos*, antioxidant, hepatoprotective activity

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by normal physiologic processes and fulfill important functions in the body at minute or moderate concentrations. ROS and RNS are necessary for the maturation process of cellular structures and can act as weapons for the host's defense system. The importance of ROS production by the immune system is clearly evident in patients with granulomatous disease. These patients have a defective membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system that precludes them from producing the superoxide anion radical (O_2^-), thereby resulting in multiple and persistent infections (*1,2*). Oxygen radicals have crucial action such as signal transduction, gene transcription, and regulation of synthesis of cyclic guanosine monophosphate (cGMP) in cells (*3,4*). Nitric oxide (NO) is a common signaling molecule and participates in virtually every one of the body's cellular and organ functions (*5*). Optimum amounts of NO produced by endothelial cells are essential for regulating the relaxation and proliferation of vascular smooth muscle cells, leukocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone, and hemodynamics (*5*). In addition, NO produced by neurons serves as a neurotransmitter (*6*).

However, the generation of even slightly larger amounts of these essential compounds during metabolism or in response to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods, and physical stress can cause massive physiologic problems by oxidation of bio-molecules (protein, amino acids, lipid, and DNA). Free radicals presumably play a major role in all pathologies. Free radicals are believed to be responsible for more than one hundred conditions like cancer, diabetes, atherosclerosis, arthritis, neuropathy, nephropathy, retinopathy, aging, compromised immunity, and cardiovascular diseases (*7-10*).

The liver has a central role in transforming and clearing chemicals and is closely related to the gastrointestinal tract, which makes it susceptible to drug toxicity, xenobiotics, and oxidative stress. Dysfunction of this organ results in impairment of energy metabolism and intracellular oxidant stress with excessive formation of ROS. CYP2E1 is a cytochrome P450 isoenzyme produced by the liver that also facilitates oxidative stress and cell injury (*11,12*). Although Kupffer cells and recruited neutrophils in the liver are part of the host-defense system, these inflammatory cells initiate additional liver injury under certain circumstances, such as when excess free radicals are present (*13-16*).

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Although the body has mechanisms to defend against the damaging properties of ROS (*17,18*), its capacity to control ROS can be overwhelmed, causing irreversible oxidative damage and various conditions that threaten the liver and other parts of the body. Several synthetic and semi-synthetic chemicals are often used to protect the liver from the detrimental effects of free radicals but they are not sufficiently effective and cause adverse reactions. Modern medicine includes many important bioactive molecules with antioxidant and hepatoprotective properties that were derived from plants. Curcumin and lycopene are used as antioxidants and are respectively obtained from the plants *Erythroxylum coca* and *Lycopersicon esculentum*. *Andrographis paniculata* and *Silybum marianum* are two important plant sources of andrographolide and silymarin, respectively, that are widely used as hepatoprotective agents (*19*). Hence, recent research has hastened to identify notable hepatoprotective agents from plant products that will reduce the harmful effects of and problems associated with free radicals while causing minimal adverse reactions. As part of ongoing research, the current study examined the ethanol extract of *Syzygium jambos* leaves for its use as a hepatoprotective agent to reduce the damage caused by ROS and RNS.

Syzygium jambos (L.), commonly known as rose apple, belongs to the family Myrtaceae and may merely be a shrub but is generally a tree found all over parts of Bangladesh, Pakistan, and India (*20,21*). It has been used in traditional medicine to treat various ailments. The fruit is regarded as a tonic for the brain and liver. The seeds are used to curb diarrhea, dysentery, and catarrh and help treat diabetes. A decoction of the leaves is applied to sore eyes, it serves as a diuretic and expectorant, and it is used to treat rheumatism. The bark contains 7-12.4% tannin. The leaf extract of *Syzygium jambos* reportedly has antinociceptive and antimicrobial activity (*22,23*). No reports have described the plant's hepatoprotective activity. Thus, the present study sought to investigate the leaves of the *S. jambos* (SJL) for its hepatoprotective activity using a rat model of CCl_4-induced liver damage and different *in vitro* antioxidation experiments.

2. Materials and Methods

2.1. *Chemicals*

1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), ferric chloride, gallic acid, and quercetin were obtained from Sigma Chemical Co., USA. Ascorbic acid and aluminium chloride were obtained from SD Fine Chem. Ltd., Biosar, India. Ammonium molybdate, methanol, sodium phosphate, concentrated H_2SO_4, Folin-Ciocalteu reagent, sodium carbonate, potassium acetate, mono-sodium phosphate, bi-sodium phosphate, potassium ferricyanide, and trichloro acetic acid were purchased from Merck, Germany.

2.2. *Preparation of extract*

An ethanolic extract of leaves was used in the present study. Mature leaves were collected in July 2009 from Rajshahi, Bangladesh. The leaves were dried in the shade and pulverized in a mechanical grinder. The powder was extracted with ethanol. The extracted solution was filtered using a clean cloth and then filter paper. The extract was concentrated first in a rotary vacuum evaporator and then in a water bath. The extracted residue was weighed and the percent yield of leaves of *S. jambos* was 9.21% w/w. The extract was then frozen prior to examination of its potential antioxidant and hepatoprotective properties.

2.3. *DPPH radical scavenging activity assay*

The free radical scavenging capacity of the extract was determined using the stable free radical DPPH (*24*). The leaf extract was mixed with 95% ethanol to prepare a stock solution (5 mg/mL). DPPH solution (0.004%, w/v) was prepared in 95% ethanol. A freshly prepared DPPH solution (0.004%, w/v) was placed in test tubes and SJL was added followed by serial dilution (1 µg to 500 µg) in every test tube so that the final volume was 3 mL. After 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DR UV-visible spectrophotometer, USA). Ascorbic acid was used as a reference standard and dissolved in distilled water to prepare a stock solution with the same concentration (5 mg/mL). A control sample of the same volume was prepared without any extract and reference ascorbic acid. A solution of 95% ethanol served as a blank. The % scavenging of the DPPH free radical was measured using the following equation:

$$\% \text{ Scavenging activity} = \text{Absorbance of the control} - \text{Absorbance of the test sample/Absorbance of the control} \times 100$$

The inhibition curve was plotted for experiments in triplicate and expressed as the % of mean inhibition ± standard deviation. IC_{50} values were obtained by Probit analysis (*25*). The IC_{50} value is the concentration of the sample required to inhibit 50% of the radical.

2.4. *Total antioxidant capacity assay*

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method in accordance with the procedure described by Prieto *et al*. (*26*). The assay is based on the reduction of Mo(VI) to Mo(V) by the extract/sample and subsequent formation of a green phosphate/Mo(V) complex at an acidic pH. A 0.3 mL extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Test tubes containing the reaction solution were incubated at 95°C for 90 min. The absorbance of the solution was then measured against a

blank at 695 nm using a spectrophotometer (HACH 4000 DU UV-visible spectrophotometer) after the solution cooled to room temperature. Ethanol (0.3 mL) in the place of extract/sample served as the blank. Antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

2.5. *Reducing capacity assay*

Reducing power of the extract was evaluated using the Oyaizu method (*27*). Different concentrations of SJL extract (125, 250, 500, and 1,000 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1% w/v). The mixture was incubated at 50°C for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid solution was added to each test tube and the mixture was centrifuged at 3,000 rpm for 10 min. Five mL of the upper layer solution was mixed with 5 mL of distilled water and 1 mL of ferric chloride solution (0.1%, w/v) and the absorbance was measured at 700 nm. The reducing power of the extract was linearly proportional to the concentration of the sample. Ascorbic acid served as the reference standard. Phosphate buffer (pH 6.6) served as the blank solution.

2.6. *Determination of total phenol content*

Total phenol content in the extract was determined with Folin-Ciocalteu reagent. Extract (200 µg/mL) was mixed with 400 µL of the Folin-Ciocalteu reagent and 1.5 mL of 20% sodium carbonate. The mixture was shaken thoroughly and brought to 10 mL using distilled water. The mixture was allowed to stand for 2 h. The absorbance at 765 nm was then determined. The total phenol content in SJL was then determined as the mg of gallic acid equivalent using equations that were obtained from a standard gallic acid graph (*28*).

2.7. *Determination of total flavonoid content*

The total flavonoid content was determined using a method previously described by Kumaran and Karunakaran (*29*). One mL of plant extract in ethanol (200 µg/mL) was mixed with 1 mL aluminium trichloride in ethanol (20 mg/mL) and a drop of acetic acid and then diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min. Blank samples were prepared from 1 mL of plant extract and a drop of acetic acid and then diluted to 25 mL with ethanol. The total flavonoid content was determined using a standard curve for quercetin (12.5-100 µg/mL) and was expressed as mg of quercetin equivalent (QE/g of extract).

2.8. *Hepatoprotective activity*

The hepatoprotective activity of SJL extract was determined using a rat model of carbon tetrachloride-induced hepatotoxicity. After seven days of acclimatization, rats were divided into four groups consisting of three rats each. Treatment lasted for 8 days. Group I served as the normal control and received only normal saline (1 mL/kg, *i.p.*) for eight days. Group II served as the toxic control and was administered carbon tetrachloride in liquid paraffin (CCl_4:liquid paraffin 1:2; 1 mL/kg, *i.p.*) once every 72 h. Group III served as the positive control and was administered silymarin (25 mg/kg/day, *p.o.*) along with carbon tetrachloride in liquid paraffin (CCl_4:liquid paraffin 1:2; 1 mL/kg, *i.p.*) once every 72 h. Group IV was administered SJL (300 mg/kg, *p.o.*) daily and carbon tetrachloride in liquid paraffin (CCl_4:liquid paraffin 1:2; 1 mL/kg, *i.p.*) once every 72 h. Twenty-four h after the last dose, blood was taken from the retro-orbital plexus under sodium phenobarbital anesthesia and rats were dissected to remove the liver. Before blood was collected, the syringe was ringed with heparin to prevent hemolysis/clotting. The blood samples were then centrifuged at 2,500 rpm at 37°C to separate serum and were used to estimate the biochemical markers of liver damage, *i.e.* SGOT, SGPT (*30,31*), ALP (*32*), bilirubin (*33*), and total protein levels (*34*).

2.9. *Statistical analysis*

Linear regression analysis was used to calculate IC_{50} values wherever needed. All results are expressed as average ± SEM. Data were statistically evaluated in InStat software using one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *DPPH radical scavenging activity*

A DPPH assay is one of the most widely used methods of screening for the antioxidant activity of plant extracts. DPPH is a stable, nitrogen-centered free radical that produces a violet color in ethanol solution. When DPPH encounters proton donors such as antioxidants, it is reduced to a yellow product, diphenyl picryl hydrazine, and then absorbance decreases. The antioxidant activity of an ethanol extract of SJL was evaluated by measuring its scavenging capacity for the DPPH free radical; this capacity was expressed using the IC_{50} value. A lower IC_{50} value indicates an extract with greater scavenging activity. The extract had dose-dependent activity, *i.e.* DPPH scavenging activity increased proportionate to the increase in concentration of the extract. Results are shown in Figure 1. The IC_{50} value of the extract was 14.10 µg/mL while that for reference ascorbic acid was 4.87 µg/mL. These results indicate that the extract had definite free radical scavenging activity in comparison to ascorbic acid.

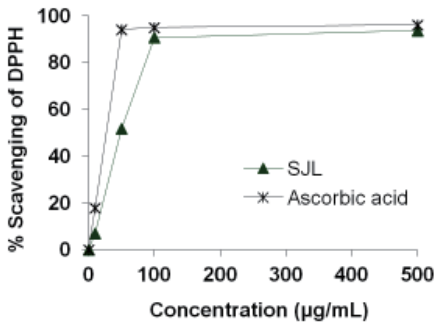

Figure 1. DPPH radical scavenging activity of the ethanolic extract of *S. jambos* leaves. Values are the average of experiments in triplicate and are expressed as mean ± standard deviation.

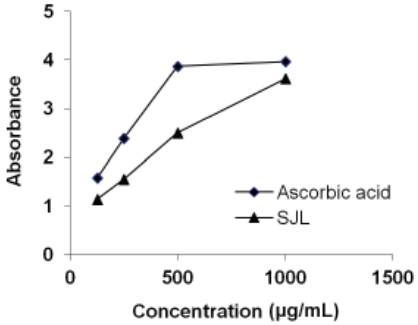

Figure 2. Reducing power of *S. jambos* leaves and ascorbic acid.

Table 1. Effect of ethanol extract of *S. jambos* leaves and silymarin on serum biochemical parameters in CCl_4-induced liver damage in rats

Treatment group	Serum biochemical parameters					Liver weight (g)
	SGPT (U/L)	SGOT (U/L)	ALP (KA)	Bilirubin (mg/dL)	Total protein (mg/dL)	
Normal (control)	22.1 ± 0.33	38.8 ± 0.39	19.26 ± 0.04	1.06 ± 0.016	12.14 ± 0.18	5.96 ± 0.18
CCl_4	74.3 ± 0.51	85.4 ± 0.39	68.12 ± 0.12	5.68 ± 0.027	7.67 ± 0.064	9.57 ± 0.31
SJL	41.7 ± 0.60	66.9 ± 0.34	49.29 ± 0.09	4.59 ± 0.019	8.12 ± 0.06	7.33 ± 0.27
Silymarin	26.8 ± 0.33	46.0 ± 0.34	24.47 ± 0.13	2.33 ± 0.036	11.08 ± 0.08	6.98 ± 0.42

Values are mean ± STD and each group contained three rats. Drug treatment lasted for 8 days. $^{\#}$ $p < 0.001$ for the CCl_4-treated group compared to the normal control group; * $p < 0.05$ for experimental groups compared to the control group. Significance was tested using one-way ANOVA followed by a post hoc Dunnett's test.

3.2. *Total antioxidant capacity*

The total antioxidant capacity of SJL was determined using a calibration curve for ascorbic acid (y = 0.0043x + 0.1503) and was expressed as the ascorbic acid equivalent (AE). Total antioxidant capacity was 335.70 ± 65.77 mg AE/gm of extract.

3.3. *Reducing capacity*

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (*35*). Reducing properties are generally associated with the presence of reductones, which have been shown to have antioxidant action by breaking the free radical chain reaction by donating a hydrogen atom (*36,37*). They can react with free radicals to convert them into more stable products. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation.

The reducing capacity of SJL was investigated using Fe^{3+}-Fe^{2+} transformation. The presence of reductones causes the reduction of Fe^{3+}/ferricyanide complex to the Fe^{2+} form. This Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The extract had significant reducing activity compared to ascorbic acid and this activity increased proportionate to the increase in concentration of the extract, as shown in Figure 2. An increase in the reaction mixture's absorbance indicates an increase in the reducing capacity of the sample.

3.4. *Total phenol content and flavonoid content*

The Folin-Ciocalteu reagent was used to estimate total phenols present in the extract and this value was expressed as gallic acid equivalents (GAE). The total phenolic content of the sample, calculated on the basis of the standard curve for gallic acid, was found to be 161.78 ± 11.78 mg GAE/gm of SJL extract. The total flavonoid content of SJL was 1,033.37 ± 62.05 mg of quercetin equivalent per gram of extract.

3.5. *Hepatoprotective activity*

The hepatoprotective activity of the crude ethanol extract at a dose of 300 mg/kg body weight in rats with carbon tetrachloride-induced damage is shown in Table 1. For comparison, the table also shows the untreated group (control), carbon tetrachloride-treated group (induction control), and the group treated with the drug (silymarin). The control group (I) had serum SGPT of 22.1 ± 0.33 U/L, SGOT of 38.8 ± 0.39 U/L, ALP of 19.26 ± 0.04 mg/dL, total bilirubin of 1.06 ±

0.016 mg/dL, total protein of 12.14 ± 0.18 KA, and liver weight of 5.96 ± 0.18 g. In the group with CCl_4-induced liver damage (II), serum SGPT increased to 74.3 ± 0.51 U/L, SGOT increased to 85.4 ± 0.39 U/L, ALP increased to 68.12 ± 0.12 mg/dL, total bilirubin increased to 5.68 ± 0.027 mg/dL, total protein increased to 7.67 ± 0.064 KA, and liver weight increased to 9.57 ± 0.31 g. Administration of SJL extract in rats with CCl_4-induced liver damage resulted in gradual normalization of SGPT, SGOT, ALP, total bilirubin, total protein, and liver weight ($p < 0.001$ compared to the CCl_4-treated group).

4. Discussion

There is growing evidence that the altered production and spatiotemporal distribution of ROS/RNS causes deleterious oxidative/nitrosative stress (*38*). This condition leads to the interaction of ROS/RNS and bio-molecules (protein, amino acids, lipid, and DNA) and interferes with the expression of a number of genes and signal transduction pathways. Thus, ROS/RNS play a key role in damage to cell structures as well as various diseases and aging (*39*). The liver is one of the most important organs in the body. It plays a vital role in regulating, synthesizing, storing, secreting, transforming, and breaking down many different substances in the body. The liver plays a central role in transforming and clearing chemicals and hence it is most susceptible to the free radicals from these agents (*40*). The body has several mechanisms to counteract oxidative/nitrosative stress with antioxidants, either naturally generating them in situ (endogenous antioxidants) or obtaining them externally through foods (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects, and to help prevent disease.

Results of the current study indicated that the ethanol extract of SJL has significant antioxidant capacity, obvious reducing capacity, and definite DPPH radical scavenging activity. These pharmacological effects of the extract may at least in part be due to phenols and flavonoids components that were found in the SJL ethanol extract. Phenols are ubiquitous secondary metabolites in plants and have a wide range of therapeutic uses because of their antioxidant, antimutagenic, anticarcinogenic, and free radical scavenging activity (*41*). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching single and triplet oxygen, or decomposing peroxides (*42*). Flavonoids are a group of polyphenolic compounds with known properties, which include free radical scavenging and inhibition of hydrolytic and oxidative enzymes (*43*). Furthermore, these compounds have a strong affinity for iron ions (which are known to catalyze many processes and lead to the appearance of free radicals), so their antiperoxidative activity could also be attributed to a concomitant ability to chelate iron. Therefore, the phenol and flavonoid components in the SJL ethanol extract may have contributed directly to antioxidant action noted in this study.

In most developing countries, there is a high incidence of viral hepatitis. Identification of an efficient hepatoprotective drug derived from natural sources is an urgent necessity. The changes associated with CCl_4-induced liver damage are similar to those of acute viral hepatitis (*44*). CCl_4 is therefore a useful tool for inducing hepatic damage in experimental animals. The hepatotoxicity of CCl_4 is the result of its reductive dehalogenation. It is catalyzed by cytochromic P450 to produce the highly reactive metabolite trichloromethyl ($CCl_3\cdot$) free radical. This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical ($CCl_3OO\cdot$). These free radicals bind covalently to cellular proteins or lipids or extract a hydrogen atom from an unsaturated lipid, thereby initiating lipid peroxidation and consequently leading to liver damage. A substantial increase in the level of serum marker enzymes (SGOT, SGPT, and ALP) and total bilirubin was noted in the CCl_4 control group. The return of elevated levels of serum enzymes to near normal values in groups treated with the leaf extract or standard silymarin is an indication of the stabilization of the plasma membrane and the repair of hepatic tissue damage caused by CCl_4. A decrease in the level of total proteins in rats with CCl_4-induced liver damage is attributed to damage primarily in the endoplasmic reticulum. This results in a loss of P450 and subsequent decrease in protein synthesis. The rise in protein levels in treated groups suggests the stabilization of the endoplasmic reticulum and subsequent protein synthesis. In addition, CCl_4 led to a significant increase in liver weight because it blocks the secretion of hepatic triglycerides in plasma (*45*). Silymarin and the extract were found to prevent an increase in liver weight in rats. These results suggest that the ethanol extract of SJL offers hepatoprotection by reducing damage or by preserving normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin such as CCl_4. This finding indicates that SJL has protective action *in vivo*.

Results of the present study indicate that an ethanol extract of SJL has potential antioxidant activity *in vitro* and hepatoprotective activity *in vivo.* However, further studies are needed to examine underlying mechanisms of antioxidant activity *in vitro* and hepatoprotective activity *in vivo*. Studies also need to isolate the active compound(s) responsible for this pharmacological activity.

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Antimicrobial screening of some Egyptian plants and active flavones from *Lagerstroemia indica* leaves

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ABSTRACT: One hundred and twenty four plant extracts were evaluated for their antimicrobial activity against four pathogenic bacteria (*Staphylococcus aureus* (ATCC 8095), *Salmonella enteritides* (ATCC 13076), *Escherichia coli* (ATCC 25922), and *Listeria monocytogenes* (ATCC 15313)) and *Candida albicans* yeast (ATCC 10231) using the disk diffusion and broth microdilution methods. Of the plant extracts, fourteen exhibited antimicrobial activity against two or more of the five microorganisms tested. Only the methanol extract of *Lagerstroemia indica* leaves exhibited antimicrobial activity against all pathogenic bacteria and *C. albicans* yeast that were tested. Purification of the methanol extract of *L. indica* leaves using antimicrobial assay-guided isolation yielded one pure active compound. The chemical structure of the isolated active compound was found to be '4-methoxy apigenin-8-*C*-*β*-D-glucopyranoside; cytisoside according to detailed spectroscopic analysis of its nuclear magnetic resonance and mass spectrometry data. The compound exhibited antimicrobial activity against *C. albicans* (minimum lethal concentration (MLC): 32 µg/mL), *S. aureus* (MLC: 16 µg/mL), *S. enteritides* (MLC: 16 µg/mL), *E. coli* (MLC: 16 µg/mL), and *L. monocytogenes* (MLC: 16 µg/mL). The present study found that the methanol extract of *L. indica* leaves holds great promise as a potential source of beneficial antimicrobial components for different applications.

Keywords: Pathogenic bacteria, *Candida albicans*, *Lagerstroemia indica*, flavones, antimicrobials

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1. Introduction

Secondary metabolites produced by plants constitute a major source of bioactive substances. Over the past two decades, the scientific interest in these plant metabolites has increased as part of the search for new therapeutic agents, partly due to the increasing resistance of microorganisms to most antimicrobial drugs currently used in medicine and agriculture (*1*). In addition, the need for safer agrochemicals with less environmental and mammalian toxicity is a major concern. In an attempt to discover new chemical classes of antimicrobial drugs that could resolve these problems, a wide range of plant extracts has been examined for antimicrobial properties (*2,3*).

Food products are susceptible to contamination and spoilage by bacteria. More than 90% of the cases of food poisoning or food-related infection each year are caused by Gram-positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* or Gram-negative bacteria such as *Escherichia coli* and *Salmonella enteritidis* (*4-7*). In recent years, the effects of plant extracts and phytochemicals on food pathogenic bacteria have been studied (*8-11*). Candidiasis is an infection caused by *Candida* yeast, especially *Candida albicans*, and is associated with skin infection and other health problems. Treatment of candidiasis can be problematic due to the limited number of effective antifungal drugs, toxicity of the available antifungal drugs, resistance of *Candida* to commonly-used antifungal drugs, relapse of *Candida* infections, and the high cost of antifungal drugs (*12*). The investigation of natural products for activity against *Candida* species has therefore increased in the last 10 years, with approximately 258 plant species from 94 families having been investigated (*13*).

The present study evaluated the chloroform and methanol extracts of 124 plant species grown in Egypt (belonging to 56 plant families) for their antimicrobial activity against *C. albicans* yeast and four food pathogenic bacteria (*S. aureus*, *S. enteritidis*, *E. coli*, and *L. monocytogenes*). As the methanol extract of the leaves of *Lagerstroemia indica* exhibited significant antimicrobial activity against all five microorganisms, bioactivity-guided separation of the methanol extract of *L. indica* leaves was used to isolate and identify the active compound.

2. Materials and Methods

2.1. *Chemicals and plant materials*

All chemicals and reagents used in this study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA), BDH (Dorset, England) or Fluka Chemie Co. (Buchs, Switzerland).

Leaf samples from a total of 124 plant species belonging to 56 families were collected during April and May 2008 from El-Shrouk farm on the Cairo-Alexandria desert road, 72 km north of Cairo. The botanical identification of the collected specimens was confirmed by Dr. T. Labeb, Herbarium of Orman Garden, Horticulture Research Institute, Giza, Egypt. A voucher specimen of each plant was deposited in the herbarium of the Biochemistry Department, Faculty of Agriculture, Fayoum University. The leaves were cleaned, air-dried in the shade, and then powdered to pass through 24 mesh using a laboratory mill. Powdered material was stored in an air-tight container at room temperature (28 ± 2°C) and protected from light until use.

2.2. *Detection and identification methods*

Analytical thin-layer chromatography (TLC) was carried out on Merck precoated silica gel plates (F245, 0.25 mm thickness). Analytes were visualized under ultraviolet (UV) light (254 and 365 nm) by spraying 30% H_2SO_4 in methanol followed by heating at 105°C for 5 min, by spraying $AlCl_3$ reagent to detect flavonoids, or by spraying naphthoresorcinol/phosphoric acid followed by heating at 105°C for 10 min to detect sugars. The purified compounds were preliminary characterized phytochemically for the presence of saponins, flavonoids, alkaloids, tannins, and glycosides using TLC according to methods described previously (*16*).

^{1}H, ^{13}C nuclear magnetic resonance (NMR) and heteronuclear multiple bond correlation (HMBC) spectra were recorded in deutero-methanol (CD_3OD) on a Bruker DRx 400 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) at 400 MHz for ^{1}H and 100 MHz for ^{13}C. UV spectra were recorded on a Cecil 3000 series spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). Mass spectra were recorded on a GC/MS Qp 100 Ex Shimadzu Mass spectrometer (Shimadzu, Kyoto, Japan) at 70 eV.

2.3. *Preparation of plant extracts*

A known amount of air-dried powdered leaves from each plant was extracted with chloroform at room temperature (28 ± 2°C). This procedure was repeated at least five times and until the organic solvent remained colorless. The same chloroform-extracted samples were then extracted with methanol at least five times or until the solvent remained colorless. The extracts obtained were filtered using Whatman No. 1 filter paper and the combined extract (filtrate) was evaporated to dryness in a rotary evaporator at 45°C. The antimicrobial activity of the dry residue was then assayed.

2.4. *Isolation of bioactive compound(s) from L. indica leaves*

The bioactive methanol extract of *L. indica* leaves was subjected to chromatography to isolate antimicrobial component(s) as follows. Thirteen grams of extract residue in methanol were loaded onto a chromatographic column (5 cm × 100 cm) packed with silica gel (230-400 mesh, 700 g, Merck & Co. Inc., Whitehouse Station, NJ, USA) and eluted with a gradient of chloroform:methanol (85:15, 70:30, 50:50, and 30:70, v/v; 2 L for each eluent). Twenty fractions (100 mL) of each eluent were collected and assayed with TLC. Based on their similarities in TLC, the collected fractions were combined into 11 fractions that were further tested for antimicrobial activity. Two fractions (**8** and **9**) exhibited strong antimicrobial activity. These fractions (**8**, 1.5 g and **9**, 2.9 g) eluted between 400-900 mL and 1,000-2,000 mL of chloroform:methanol (50:50), respectively. Fractions **8** and **9** were subjected to solid phase extraction using a C_{18} cartridge (Waters, Milford, MA, USA) and subsequently chromatographed on a Sephadex LH20 (GE Healthcare-Bio-Sciences, Piscataway, NJ, USA) column (2.7 cm inner diameter × 60 cm, 50 g resin) using methanol as the mobile phase. The eluates were combined into eight fractions (A-H) on the basis of similar TLC profiles. Fraction E (960 mg) contained the most antimicrobial activity and was further purified on a silica gel column (1.5 cm inner diameter × 60 cm; 50 g) with chloroform:methanol:water (60:40:5, v/v) followed by purification on a Sephadex LH20 column (1.6 cm inner diameter × 40 cm; 20 g) with methanol as the eluent to yield 543 mg of pure active compound.

2.5. *Test organisms and the agar disk diffusion method*

The four food pathogenic bacteria (*S. aureus* (ATCC 8095), *S. enteritidis* (ATCC 13076), *E. coli* (ATCC 25922), *L. monocytogenes* (ATCC 15313)) and *C. albicans* (ATCC 10231) were obtained from the Department of Agricultural Microbiology, Faculty of Agriculture, Fayoum University, El-Fayoum, Egypt. Stock cultures of bacteria were maintained on nutrient agar slants at 4°C, and Candida yeast was maintained on potato dextrose agar slants at 4°C. Bacterial and yeast cultures were sub-cultured in Petri dishes prior to testing.

The disk diffusion method (*14*) was used to assay the antimicrobial activity of plant extracts. Three sterilized filter paper discs (6 mm) were soaked with each plant extract (1 g/10 mL) and dried at 40°C. The dried disks were transferred to the surface of the inoculated plates in triplicate. Plates with pathogenic bacteria were then incubated at 37°C for 24-48 h and plates with *Candida* yeast were incubated at 30°C for 48-72 h. Afterwards,

bioactivity was determined by measuring the diameter of inhibition zones (DIZ) around each disk in mm. Solvent without test compounds was used as a control.

2.6. *Determination of minimum lethal concentrations (MLC)*

The MLC of leaf extracts with potential antimicrobial activity and their isolated active constituent(s) were determined using the broth micro-dilution method (*15*). The lowest concentration of a tested extract or compound resulted in a viable count of less than 0.1% of the original inoculum (1×108 colony-forming units per milliliter, cfu/mL, as compared to the turbidity of the McFarland 0.5 standard). Ampicillin and fluconazole served as standards for comparison in antibacterial and antifungal tests, respectively.

3. Results

3.1. *Antimicrobial activity of plant leaf extracts*

The chloroform and methanol extracts of a number of Egyptian plants (124 plant species) belonging to 56 plant families were evaluated for their antimicrobial activity against five pathogenic organisms (*C. albicans* yeast and four food pathogenic bacteria: *S. aureus*, *S. enteritidis*, *E. coli*, and *L. monocytogenes*) using a disk diffusion assay. The methanolic extracts of fourteen plant species exhibited significant antimicrobial activity against two or more of the five pathogenic microorganisms that were tested. The diameter of inhibition zones (DIZ) and the MLC of the promising plant extracts are shown in Table 1. The data indicated that the methanol extracts of these plants exhibited varying levels of antimicrobial activity against the test microorganisms.

The results in Table 1 show that among the fourteen promising plant extracts tested, extracts of *Pelargonium odoratissimum*, *Pelargonium zonale*, and *Rosa spp* were active against *E. coli*, *S. aureus*, *S. enteritidis*, and *L. monocytogenes*. Seven extracts were found to be only active against *E. coli*, *S. enteritidis*, and *L. monocytogenes*. This variation in activity may be due to the intrinsic tolerance of microorganisms and the nature and combination of phytocompounds present in these extracts (*17*). Only a leaf extract of *L. indica* was active against all five of the pathogenic microorganisms tested. Of the bacterial strains tested, *E. coli* was found to be the most sensitive and was inhibited by all plant extracts that exhibited antimicrobial activity.

3.2. *Purification of an antimicrobial compound from methanolic L. indica leaf extract*

Methanolic *L. indica* leaf extract had both bactericidal and fungicidal activity against the five microorganisms tested, with an MLC ranging between 60 and 120 µg/mL. This is similar to the MLC of standard antibiotics (fluconazole and ampicillin ranging from 24 to 128 µg/mL). The results clearly showed that the methanol extract of *L. indica* was the most effective plant extract. It inhibited the growth of both Gram-negative and Gram-positive bacteria that were tested and also of the yeast *C. albicans*, which has a sophisticated mechanism of resistance to many drugs (*18*). Because the methanolic extract of *L. indica* had broad-spectrum antimicrobial action against all five pathogenic organisms tested, this extract was selected for phytochemical analysis and isolation of its bioactive constituent(s).

Table 1. Evaluation of the antimicrobial potential of promising plant extracts and the active compound from *L. indica* (cytisoside) indicated by the diameter of inhibition zones (DIZ, mm) and the minimum lethal concentration (MLC, µg/mL) against pathogenic organisms: *C. albicans* yeast and four bacteria, *i.e. L. monocytogenes*, *S. aureus*, *S. enteritidis*, and *E. coli*

Plant species	Plant family	*L. monocytogenes*		*S. enteritidis*		*S. aureus*		*E. coli*		*C. albicans*	
		DIZ	MLC	DIZ	MLC	DIZ	MLC	DIZ	MLC	DIZ	MLC
Yucca desmettiana	Agavaceae	–	–	–	–	–	–	24	> 120	20	> 120
Dracaena marginata	Agavaceae	–	–	–	–	–	–	21	120	23	> 120
Schinus terebinthifolius	Anacardiaceae	23	> 120	31	120	–	–	27	> 120	–	–
Euonymus japonica	Celastraceae	15	120	15	120	–	–	20	120	–	–
Conocarpus erectus	Combretaceae	20	120	24	60	–	–	34	120	–	–
Acalypha marginata	Euphorbiaceae	17	120	25	30	–	–	30	120	–	–
Pelargonium odoratissimum	Geraniaceae	18	120	20	120	35	120	30	120	–	–
Pelargonium zonale	Geraniaceae	23	120	32	30	32	> 120	30	30	–	–
Asparagus plumosus	Liliaceae	–	–	–	–	–	–	15	> 120	18	> 120
Lagerstroemia indica	Lythraceae	23	120	30	60	30	60	26	120	20	120
Myrtus communis	Myrtaceae	23	> 120	31	120	–	–	20	120	–	–
Punica granatum	Punicaceae	17	120	21	30	–	–	28	120	–	–
Rosa spp	Rosaceae	20	120	29	120	34	120	32	120	–	–
Cestrum diurnum	Solanaceae	12	> 120	–	–	–	–	16	> 120	21	120
Cytisoside (*Control*)		–	16	–	16	–	16	–	16	–	32
Fluconazole (*Control*)		–	–	–	–	–	–	–	–	–	> 128
Ampicillin (*Control*)		–	24	–	24	–	24	–	24	–	–

Phytochemical examination of the extract indicated the presence of phenolic compounds, flavonoids, triterpenoids, alkaloids, and glycoside compounds. One or more of these secondary metabolites may be responsible for antimicrobial activity (*19*).

Bioactivity-guided separation of the methanol extract of the dried leaves of *L. indica* resulted in the isolation of one chromatographically pure compound. The isolated compound had antimicrobial activity against the five human pathogenic microorganisms tested (Table 1). The MLC ranged between 16 and 32 µg/mL, indicating greater activity than that of the standard antibiotics used as positive controls in this experiment. Therefore, this compound was at least in part responsible for the antimicrobial activity of leaves of *L. indica.*

3.3. *Characterization and identification of the active compound*

The purified compound was obtained as a yellow amorphous solid that resulted in a positive color reaction with $AlCl_3$ reagent on TLC, suggesting a flavonoid. Electron ionization mass spectrometry resulted in a molecular ion peak [M^+] at 434 m/z. This finding, together with ^{1}H- and ^{13}C-NMR spectroscopic data, suggested a molecular formula of $C_{21}H_{22}O_{10}$. The UV spectrum had absorption maxima at λ 270 (band II) and 333 nm (band I), which are characteristic absorption bands of the flavone skeleton (*20*). No shift in band I of this compound was observed after the addition of $AlCl_3$/HCl, suggesting the formation of a hydroxyl-keto complex at 5-OH and the absence of an *O*-dihydroxyl group in the β-ring (*21*). The ^{1}H-NMR spectrum of this compound displayed signals characteristic of a methoxy group (δ_H 3.5 (3H)) and six aromatic hydrogens (δ_H 6.4 (H), 6.5 (H), 6.83 (2H), and 7.75 (2H)) in the low field region. The appearance of four aromatic proton signals at δ 6.83 (2H, d, J = 8.8 Hz, H-'3, H-'5) and 7.75 (2H, d, J = 8.8 Hz, H-'2, H-'6) along with a characteristic pattern for an A2B2 system confirmed the substitution of the β-ring at the '4 position. The proton signals at δ_H 6.4 (1H, s) and 6.5 (1H, s) were attributed to H-3 based on HMBC and compared to reported data (*20,22*). The presence of a free 7-hydroxyl group was noted based on the appearance of a bathochromic shift in the UV spectrum upon the addition of sodium acetate. The position of the methoxy group at the '4-*O*-position of the flavone moiety was confirmed by the heteronuclear correlation according to the HMBC spectrum and the UV spectrum data in the presence of NaOMe. Therefore, the flavone moiety of this compound was determined to be '4-methoxy apigenin (acacetin). The presence of *β*-D-glucopyranose as the sole sugar moiety was noted based on NMR spectra (Table 2) given the appearance of only one anomeric proton signal at δ 4.07 (d, J = 7.6 Hz), five glucosyl protons (δ_H 3.08-3.8m) in the ^{1}H-NMR spectrum, and the appearance of one anomeric carbon atom at δ 104.68 ppm and five glucosyl carbons (75.01, 79.76, 71.56, and 62.15 ppm; C-2 to C-6) in the ^{13}C-NMR spectrum. The position of the C-glucose moiety at the C-8-position was noted based on the HMBC spectrum and by comparison to reported data (*20*). Thus, the structure of this compound (Figure 1) was found to be '4-methoxy apigenin (acacetin)-8-*C*-*β*-D-glucopyranose, which was previously reported as cytisoside (*20*).

4. Discussion

The present study identified '4-methoxy apigenin (acacetin)-8-*C*-*β*-D-glucopyranose (cytisoside) as the active antimicrobial component of methanolic *L. indica* leaf extract. This component had both antibacterial and antifungal activity. To the extent known, no previous studies isolated this active compound from the leaves of *L. indica*. Thus, this is the first study to isolate and identify an active flavone glycoside from *L. indica*,

Table 2. NMR data for the active isolated compound in CD_3OD

Atom No.	δ_C	^{13}C	^{1}H
2	C	162.80	–
3	CH	103.50	6.50 (s)
4	CO	184.06	–
5	C	164.88	–
6	CH	95.24	6.40 (s)
7	C	165.04	–
8	C	109.20	–
9	C	158.73	–
10	C	105.20	–
'1	C	123.13	–
'2	CH	129.46	7.75 (d, J = 8.8)
'3	CH	117.04	6.83 (d, J = 8.8)
'4	C	166.20	–
'5	CH	117.04	6.83 (d, J = 8.8)
'6	CH	129.46	7.75 (d, J = 8.8)
	OCH_3	57.29	3.5 (s)
"1	CH	104.68	4.07 (d, J = 7.6)
"2	CH	75.01	3.26
"3	CH	79.76	3.40
"4	CH	71.32	3.08
"5	CH	77.56	3.21
"6	CH_2	62.15	3.68, 3.80

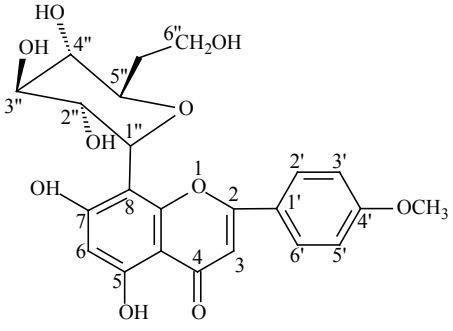

Figure 1. Structural formula of the active isolated compound ('4-methoxy apigenin-8-*C*-*β*-D-glucopyranoside; cytisoside).

i.e. cytisoside, with antimicrobial activity against the five pathogenic microorganisms (*C. albicans* yeast and four bacteria: *S. aureus*, *S. enteritidis*, *E. coli*, and *L. monocytogenes*) tested. Comparison revealed that the bioactive compound had greater antimicrobial activity than both the crude extract of *L. indica* and standard antibiotics (fluconazole and ampicillin).

Flavonoids are ubiquitous in photosynthesizing cells and therefore occur widely in the plant kingdom (*23*). Various flavonoids have been reported to possess a wide range of biological action, such as antimicrobial or antioxidant activity (*19*). Moreover, several groups of researchers have noted a synergy between biologically active flavonoids and existing chemotherapeutics (*24*). Flavonoids isolated from plant extracts are reported to have antimicrobial activity against food pathogenic bacteria and against *C. albicans*; these include compounds such as apigenin (*25,26*), quercetin and quercetin glycosides (*27,28*), luteolin (*29*), kaempferol (*30*), and flavan derivatives. Their remarkable activity is attributed to the inhibition of nucleic acid synthesis (*31*), inhibition of energy metabolism (*32*), and inhibition of cytoplasmic membrane function (*33*). Flavonoids are likely to have minimal toxicity because flavonoids are widely distributed in edible plants and beverages and have previously been used in traditional medicine.

The present study found that the methanol extract of *L. indica* leaves holds great promise as a potential source of beneficial antimicrobial components for different applications (foods and pharmaceuticals) and could resolve the problems of drug resistance and the harmful effects of synthetic compounds. However, further *in vivo* studies are needed to investigate the pharmacological and toxicological properties of *L. indica* extract before it can be considered as a new antimicrobial ingredient for the nutraceutical or functional food market.

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Original Article

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Optimization of cell-wall skeleton derived from *Mycobacterium bovis* BCG Tokyo 172 (SMP-105) emulsion in delayed-type hypersensitivity and antitumor models

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ABSTRACT: Cell-wall skeleton prepared from *Mycobacterium bovis* BCG (BCG-CWS) is known as a potent adjuvant and has been shown to possess antitumor activity in many non-clinical and clinical studies. As there are no approved BCG-CWS formulations for cancer therapy, we investigated the potential for cancer immunotherapy of SMP-105, our originally produced BCG-CWS. For optimizing SMP-105 emulsion, we compared the effects of drakeol- and squalane-based SMP-105 emulsions on IFN-γ production in rats and evaluated their ability to induce skin reaction in guinea pigs. Both emulsions had the same activity in both experiments. We selected squalane as base material and produced two types of squalane-based formulations (vialed emulsion and pumped emulsion) that can easily be prepared as oil-in-water emulsions. Although the vialed emulsion showed the same pattern of distribution as a usual homogenized emulsion, the pumped emulsion showed more uniform distribution than the other two emulsions. Whereas both emulsions enhanced strong delayed type hypersensitivity (DTH) reaction in a mouse model, the pumped emulsion induced slightly smaller edema. Data on oil droplet size distribution suggest that few micrometer oil droplet size might be appropriate for oil-in-water microemulsion of SMP-105. The antitumor potency of SMP-105 emulsion was stronger than that of some of the launched toll-like receptor (TLR) agonists (Aldara cream, Picibanil, and Immunobladder). Aldara and Picibanil showed limited antitumor effectiveness, while Immunobladder had almost the same effect as SMP-105 at the highest dose, but needed about 10 times the amount of SMP-105. These findings first indicate that SMP-105 has great potential in cancer immunotherapy.

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Keywords: Oil-in-water emulsion, oil droplet size distribution, BCG-CWS, SMP-105

1. Introduction

With the recent approval of sipuleucel-T (Provenge®) and ipilimumab (Yervoy®) by the FDA (*1*), cancer immunotherapy seems to be well underway, and is increasingly attracting a great deal of attention. Cell-wall skeleton prepared from *Mycobacterium bovis* BCG (BCG-CWS) is known as an activator of innate immunity (*2*) and has been studied in many clinical studies (*3-6*). We have previously reported that SMP-105, a highly pure cell-wall skeleton prepared from *Mycobacterium bovis* BCG Tokyo 172 strain, exhibits potent immunostimulatory activity and strong antitumor effect in animal models (*7,8*). SMP-105 is an insoluble toll-like receptor 2 (TLR2) ligand that elicits immune reactions, including induction of interferon-γ (IFN-γ) producing cells and cytotoxic T lymphocytes (CTL), and prevents tumor growth through TLR2 (*9*). SMP-105 requires phagocytosis by macrophages or dendritic cells (DCs) for immune activation and shows different *in vitro* and *in vivo* effects from those of Pam3CSK4, a soluble TLR2 ligand (*10*). Intradermal injection of an oil droplet of emulsified SMP-105 shows that this antigen is readily engulfed by phagocytes at the draining lymph nodes (*11*).

Freund's Complete Adjuvant (FCA), an immuno-potentiator composed of inactivated and dried mycobacteria in mineral oil, is known to stimulate cell-mediated immunity, but may cause severe side effects, which effectively excludes it from clinical use. BCG-CWS on the other hand is used as oil-in-water emulsion, because the immunostimulatory effect of this adjuvant depends on its emulsion form (*12*). Antitumor activity of a drakeol-base oil-in-water emulsion in clinical research has already been reported (*13*). On the other hand, there are reports showing that BCG-CWS emulsified with squalane induces safer antitumor immunity (*14,15*). Squalane-based emulsions

and their hydrogenated derivatives have been shown as potent and safe vaccine adjuvant in preclinical and clinical studies (*16-19*). In fact, MF59^{TM}, an adjuvant emulsion based on squalane, has already been approved for human use (*20*).

Studies that investigated BCG-CWS adjuvant particle size and their phagocytosis have indicated that particles with a size ranging from 0.5-2 μm are readily engulfed by macrophages (*21,22*). Labeled squalane oil-based formulations have been shown to be taken up by macrophages and DCs at the site of injection (*23*). Ideal particles size has also been investigated for drug delivery systems and as a contributing factor to generation of immune response (*24-26*). Particles made from poly lactic-co-glycolic acid (PLGA) can be used as a delivery system that provides adjuvant activity (*27,28*). These polymeric particulate delivery systems are able to present antigens and activate both humoral and cellular responses (*29,30*). A 5 μm PLGA particle containing hepatitis B virus surface antigen elicited higher immune response than a larger particle of about 12 μm (*31*). Although we have previously shown that SMP-105 requires phagocytosis by immune cells for enhancing delayed type hypersensitivity (DTH) reaction *in vivo* (*10*), there are no studies on the correlation between oil droplet size of BCG-CWS emulsion and immune activation.

In this report, we prepared several SMP-105 emulsions and compared their DTH reaction and effects in a lymph node metastasis model. We also characterized oil droplet size distribution in each emulsion. Finally, we investigated antitumor effect of originally optimized SMP-105 emulsion and compared it to that of launched TLR agonists in clinical use.

2. Materials and Methods

2.1. *Preparation of SMP-105*

SMP-105 was prepared as previously described (*4,32*). Contamination with endotoxin was less than 0.005 endotoxin units/mg. The oil-in-water emulsion of SMP-105 (homogenized emulsion) with squalane or drakeol was prepared by homogenization with a Potter-type homogenizer as previously described (*8*). The first original SMP-105 formulation, an oil-in-water emulsion of SMP-105 (vialed emulsion), was prepared and lyophilized on the thousand-vial scale (*7,11*). The vialed emulsion can be prepared by adding only water and vortexing for several seconds. Vehicle preparation used the same formulation as the vialed emulsion, except for SMP-105. The second original SMP-105 formulation, a pumped emulsified oil-in-water emulsion of SMP-105 (pumped emulsion), was prepared by pumping with an SPG pump connector (SPG techno, Miyazaki, Japan). Oil droplet size of the uniformly-sized emulsion was modulated by changing the frequency of pumping (once to 10 times). The pumped emulsion and vialed emulsion had identical content.

2.2. *Materials*

Aldara cream (5% imiquimod) was purchased from 3M Pharmaceuticals (3M Pharmaceuticals, Minnesota, USA), and Immunobladder was purchased from Alfresa Corporation (Japan BCG Laboratory, Tokyo, Japan). Picibanil (OK-432) 5KE was purchased from KSK Co., Ltd. (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan).

2.3. *Oil droplet distribution*

Oil droplet distribution was analyzed by laser diffraction using a SALD-3000J (Shimadzu Corporation, Kyoto, Japan). Oil droplet size is reported as mean diameter.

2.4. *Cells*

Lewis lung carcinoma 3LL cell line was obtained from the Cancer Institute of the Japanese Foundation for Cancer Research (Tokyo, Japan). The 3LL tumor cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 50 μg/mL streptomycin, and 50 U/mL penicillin. To prepare inactivated 3LL cells, the cells were incubated for 20 min at 37°C in culture medium containing 200 μg/mL mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan), followed by repeated washing with sufficient culture medium. Line 10 hepatocellular carcinoma cells were implanted intraperitoneally, and a range of cell stocks were prepared and stored in liquid nitrogen. In each experiment, the cells were freshly thawed before intradermal inoculation.

2.5. *Animals*

LEW/Crj male rats and C57BL/6J female mice were purchased from Charles River Japan (Kanagawa, Japan). Strain 2 male guinea pigs were obtained from Japan SLC Inc. (Shizuoka, Japan) and used at 6 weeks of age. All animals were maintained under specific pathogen free conditions, and all animal experiments were conducted according to the guidelines of the Animal Care and Use Committee of Dainippon Sumitomo Pharma.

2.6. *Rat IFN-γ production*

The oil-in-water emulsion of SMP-105 (60 μg/0.1 mL) with squalane or drakeol was administrated into the back paws of LEW/Crj rats 3 times daily. Blood was withdrawn 6 h after the third administration, and the concentration of rat IFN-γ in the serum was determined by enzyme-linked immunosorbent assay (genzyme TECHNE, Invitrogen Japan, Tokyo, Japan).

2.7. *Skin reaction*

The oil-in-water emulsion of SMP-105 (30 μg/0.1 mL) with squalane or drakeol was intradermally administrated once into the backs of Strain 2 guinea pigs. The size of skin reactions was measured at day 7, 13, 21, 27, 34, and 39 after administration.

2.8. *DTH reaction*

DTH reaction was evaluated as previously described (*8,10*). In brief, a mixture of inactivated 3LL cells (3×10^4 cells) and the vehicle; SMP-105 (12.5 μg), was intradermally administered into the left flank region of C57BL/6J mice twice with a 7-day interval. Seven days after the second administration, inactivated 3LL cells were inoculated at 10^5 cells in 50 μL HBSS into the left footpads of the mice. Just before, and 24 h after inoculation, the thickness of the left footpad was measured using a dial gauge. Percentage footpad swelling was calculated according to the following equation: Footpad swelling (%) = (thickness of post-injected footpad (mm) – thickness of pre-injected footpad (mm))/ (thickness of pre-injected footpad (mm)) × 100.

2.9. *Antitumor effect in guinea pigs*

SMP-105 antitumor effect was evaluated as described previously (*7,11*). In brief, line 10 hepatoma cells were inoculated intradermally at 1×10^6 cells in 0.1 mL into the right thoracic flank region of guinea pigs. SMP-105 and Immunobladder or vehicle was injected intradermally into sites distal to the site of tumor inoculation on days 0, 7, and 14. Picivanil was injected intradermally into sites distal to the site of tumor inoculation on days 0, 3, 7, 10, and 14. As in clinical use, Aldara cream was applied onto the inoculation sites. Animals were sacrificed by anesthesia with a high concentration of carbon dioxide and the axillary lymph nodes were collected and weighed. For pathological study, metastasis was scored from 0 to 4 based on the area occupied by the tumor cells. Metastasis rate (%) was calculated according to the following equation: Metastasis rate (%) = the number of animals with score 1 to 4 /the total number of animals in the group × 100. Metastasis Score: 0, no tumor cells observed; 1, tumor cells found as small clusters; 2, clusters composed of a significant number of tumor cells; 3, clusters composed of a large number of tumor cells, some of which are undergoing mitosis; 4, tumor clusters occupy more than half the area of the lymph node.

2.10. *Statistical analysis*

Results from all experiments are expressed as mean ± standard deviation (SD). Significant differences in skin reaction, DTH reaction, and antitumor effects were assessed using Dunnett's multiple comparison. Only one data of DTH (Figure 3A) was assessed using T-test. Statistical analysis was performed using the SAS system for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Immunostimulatory effect of drakeol-based and squalane-based SMP-105 emulsions*

Drakeol and squalane are commonly used to emulsify BCG-CWS for clinical and research use. To determine the immunostimulatory effect of drakeol-based and squalane-based SMP-105 emulsions, we measured IFN-γ concentration in serum from rats administered each of the SMP-105 emulsions. As expected, vehicle and SMP-105 dispersed in saline had no effect on IFN-γ production. On the other hand, both SMP-105 emulsions induced equally potent IFN-γ production (Figure 1A).

Because BCG-CWS has the ability to trigger skin reaction, we measured the size of skin reactions (ulcer, edema, and induration) formed after administration of each SMP-105 emulsion. Both SMP-105 emulsions dose-dependently and significantly induced skin reactions (Figure 1B). The size of the skin reactions was about the same for both emulsions. These results suggest that drakeol and squalane have similar properties as base material for SMP-105 emulsion.

3.2. *Oil droplet size distribution of oil-in-water SMP-105 emulsions*

To characterize the prepared SMP-105 emulsions, we

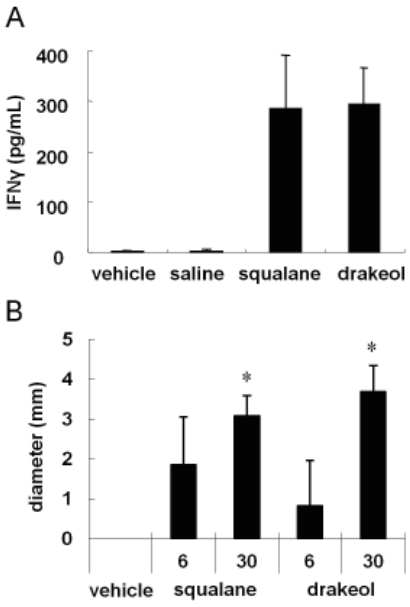

Figure 1. Immunostimulatory effect of drakeol-based and squalane-based SMP-105 emulsions. (A) IFN-γ concentration in serum from rat intradermally administrated each oil based emulsion dispersed in saline. Results are given as means ± SD of 4-5 mice. **(B)** Size of skin reactions induced by intradermal administration of each emulsion to guinea pigs. Results are given as means ± SD of maximum skin reaction size in 5 guinea pigs. * $p < 0.05$ when compared to vehicle.

measured oil droplet size distribution in each emulsion (Figure 2). Oil droplet size of SMP-105 homogenized oil-in-water emulsion with squalane and drakeol was distributed from sub-micrometer to several tens micrometers with the median about 2-3 μm (Figure 2A). Both SMP-105 emulsions (squalane-based and drakeol-based) showed the same oil droplet size distribution. The vialed emulsion had an oil droplet size close to that of the homogenized emulsion that was distributed from sub-micrometer to several tens micrometers with the median about 2-3 μm. On the other hands, the pumped emulsion showed uniform oil droplet size as compared to the homogenized and vialed emulsions (Figure 2B). Oil droplet size in the pumped emulsion differed depending on the pumping frequency (once to 10 times).

3.3. *SMP-105 emulsions enhancement of DTH in mouse model*

To investigate the immunostimulatory effect of each SMP-105 emulsion, we evaluated DTH reaction with each emulsion injection elicited by 3LL cell in mice. Mice were immunized with inactivated 3LL cell suspension admixed with each emulsion, before being injected with inactivated 3LL alone in the hind footpad, and edema was measured. As expected, the homogenized emulsion strongly enhanced swelling at the high dose (12.5 μg) of SMP-105 (Figure 3A). Although both original emulsions evoked strong footpad swelling around the high dose (12.5 μg), the edema produced by the 10 times pumped emulsion tended to be slightly weaker than that evoked by the vialed emulsion (Figures 3B and 3C). Because the pumped emulsion was composed of approximately the same material as the vialed emulsion, adequate oil droplet size seems to be required for acquired immunity against DTH reactions.

To further investigate oil droplet size, we prepared a vialed emulsion with pumping ten times that showed similar oil droplet size distribution to pumped emulsion and evaluated its induction of DTH. The pumped vialed emulsion enhanced footpad swelling induction but the swelling at highest dose was slightly weaker than that enhanced by the vialed emulsion (Figure 3D). These findings suggest that SMP-105 emulsion oil droplet size affects its immunostimulatory effect.

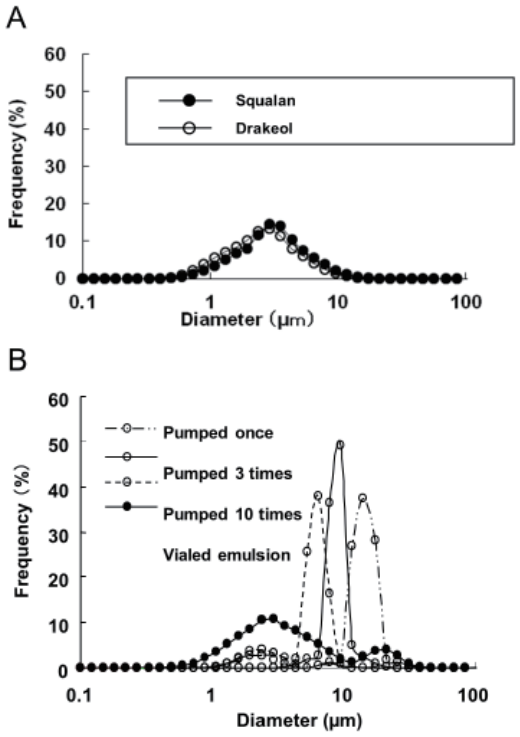

Figure 2. Analysis of oil droplet size distribution in SMP-105 emulsion. Oil droplet size distribution in SMP-105 emulsion was analyzed by laser diffraction particle size analyzer. **(A)** Squalane-based emulsion (black circle) and drakeol-based emulsion (white circle). **(B)** Vialed emulsion (black circle) and pumped emulsion (white circle) in diameter by laser diffraction particle size analyzer.

3.4. *Antitumor effect of SMP-105 emulsions in guinea pig metastasis model*

Guinea pigs were inoculated with line 10 hepatoma cells and each SMP-105 emulsion: homogenized emulsion, vialed emulsion, (10 times) pumped emulsion or vehicle. Inoculations were carried out at sites different from tumor inoculation site in order to avoid damage to tumor cells by local inflammation.

All emulsions (homogenized, vialed, and pumped) demonstrated prominent antitumor activity at 60 μg as indicated by in lymph node metastasis rate (Figures 4A and 4C) and metastasis score (Figures 4B and 4D). Growth of primary implanted tumor decreased in some animals about 2 weeks after the first dose of SMP-105.

3.5. *Antitumor effect of several TLR agonists in guinea pig model*

SMP-105 emulsions enhanced potent DTH reaction and showed antitumor activity. To confirm the antitumor activity of SMP-105, we evaluated the adequacy of the prepared SMP-105 vialed emulsion by comparing its antitumor effect on guinea pig lymph node metastasis to that of marketed TLR agonists (Aldara; TLR7 agonist, OK-432; TLR4 agonist and Immunobladder; live BCG). SMP-105 showed potent antitumor effect with a rate of lymph node metastasis about 20% (Figures 5A and 5B). Aldara inhibited lymph node metastasis by only 14.3%, but reduced the score significantly. Picivanil showed no antitumor effect on the rate of lymph node metastasis (Figures 5C and 5D). On the other hand, Immunobladder showed a dose-dependent potent antitumor effect (Figures 5E and 5F). The maximum effect of Immunobladder was almost equal to that of SMP-105, but required ten times the amount.

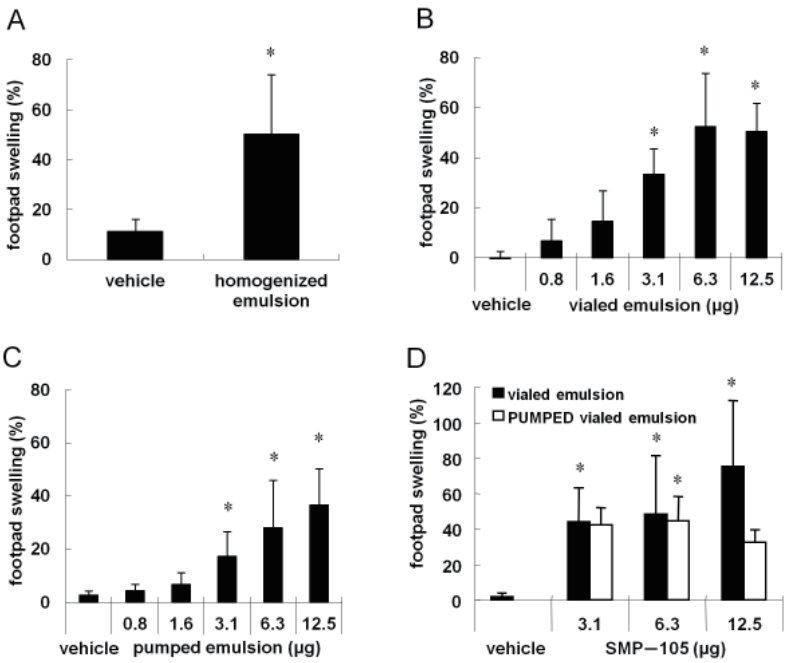

Figure 3. DTH reaction experiment with SMP-105 emulsions. A mixture of inactivated 3LL cells (3×10^4 cells) and each SMP-105 emulsion (0.8-12.5 μg) was intradermally administered into the left flank region of C57BL/6J mice twice at a 7-day interval. (**A**) Homogenized emulsion of 12.5 μg, **(B)** vialed emulsion, **(C)** pumped emulsion at each dose, **(D)** vialed emulsion and pumped vialed emulsion. Seven days after the second administration, inactivated 3LL cells were inoculated into the left footpads and swelling was monitored by measuring footpad thickness 24 h after inoculation. Relative swelling is calculated as means ± SD of 6 mice. * $p < 0.05$ when compared to vehicle.

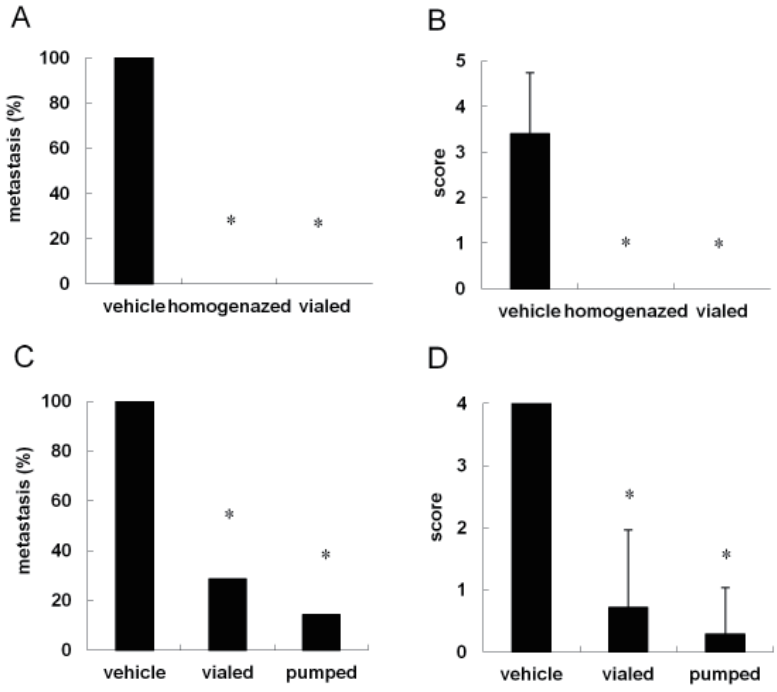

Figure 4. Antitumor effect of SMP-105 emulsions. After inoculation with line 10 hepatoma cells, SMP-105 emulsion or vehicle was injected into the same side as the tumor on days 0, 7, and 14. **(A)** Rate (%) of lymph node metastasis in the case of homogenized emulsion (60 μg) or vialed emulsion (60 μg). **(B)** Score (mean ± SD) of lymph node metastasis in the case of homogenized emulsion (60 μg) or vialed emulsion (60 μg). **(C)** Rate (%) of lymph node metastasis in the case of vialed emulsion (60 μg) or pumped emulsion (60 μg). **(D)** Score (mean ± SD) of lymph node metastasis in the case of vialed emulsion (60 μg) or pumped emulsion (60 μg). Lymph node metastasis rates are given as mean of 4-8 guinea pigs. * $p < 0.05$ when compared to vehicle.

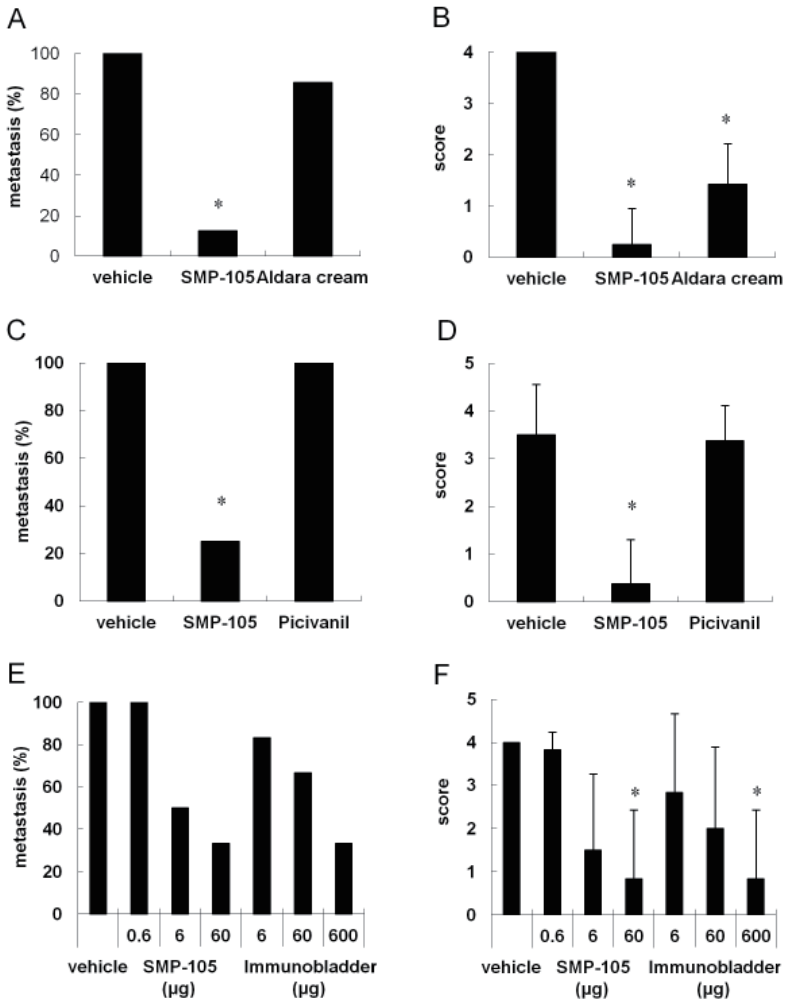

Figure 5. Antitumor effect of SMP-105 emulsion and TLR agonists on line 10 hepatoma in guinea pigs. After inoculation of line 10 hepatoma cells, SMP-105 emulsion, TLR agonist, or vehicle was injected into the same side as the tumor on days 0, 7, and 14. **(A)** Rate (%) of lymph node metastasis in the case of vialed emulsion (60 μg) or Aldara cream (100 μg). **(B)** Score (mean ± SD) of lymph node metastasis in the case of vialed emulsion (60 μg) or Aldara cream (100 μg). **(C)** Rate (%) of lymph node metastasis in the case of vialed emulsion (6 μg) or Picibanil (2KE). **(D)** Score (mean ± SD) of lymph node metastasis in the case of vialed emulsion (6 μg) or Picibanil (2KE). **(E)** Rate (%) of lymph node metastasis in the case of vialed emulsion (0.6, 6, 60 μg) or Immunobladder (6, 60, 600 μg). **(F)** Score (mean ± SD) of lymph node metastasis in the case of vialed emulsion (0.6, 6, 60 μg) or Immunobladder (6, 60, 600 μg). Lymph node metastasis rates are given as mean of 6-8 guinea pigs. * $p < 0.05$ when compared to vehicle.

4. Discussion

In this study, we prepared original BCS-CWS (SMP-105) emulsions with drakeol and squalane and showed their immunostimulatory effect and antitumor activity in animal models. We successfully generated two types of original SMP-105 formulations that can easily be made into oil-in-water emulsions. With these results, we adopted squalane as base material for SMP-105 emulsion, because of its immunostimulatory activity and its suitable properties for clinical use (Figure 1). Our original squalane-based emulsions, can easily be prepared at use, and successfully enhanced both potent DTH reaction in mouse model and antitumor activity with guinea pig lymph node metastasis model.

In this study, SMP-105 pumped emulsion induced potent footpad swelling but tended to be weaker than the vialed emulsion in enhancing DTH reaction. Interestingly, when the vialed emulsion was pumped with a connector, edema decreased compared to that observed with the vialed emulsion. We also confirmed roughly emulsified SMP-105 by voltex that showed about the same oil droplet size to once pumped emulsion enhanced DTH reaction with comparable swelling to 10 times pumped emulsion (data not shown). All emulsions were composed of almost the same material, but had different oil droplet size. The fact that both emulsions enhanced DTH reaction indicates that oil droplet size affects immunostimulatory activity. In particular, oil droplet size of the vialed emulsion seems to be an advantage for easy uptake by phagocytes. Oil droplet size distribution in the vialed emulsion ranged from sub-micrometer to over ten micrometers in diameter (Figure 2) with a mean size (about 2-3 μm) close to

the adequate size for easy uptake by macrophages (*21,22*). As previously indicated, SMP-105 requires phagocytosis by macrophages or DCs for immune activation both *in vitro* and *in vivo* (*10*). This indicates that both emulsions prepared in this study, although having different oil droplet size, were engulfed by phagocytes, suggesting that small micro droplet (about 2-3 μm) is easily devoured by phagocytes compared to large micro droplet (10 μm or larger). This consideration is consistent with the findings of other studies that show that several micrometer particles can be taken by phagocytes, but induce significant immune response (*21,22,31*). Because it is difficult to evaluate SMP-105 emulsion *in vitro*, we could not show in this study oil droplet uptake quantitatively and directly. Further studies are required to elucidate the mechanism of oil droplet uptake.

Our experiment on DTH reaction showed slight difference in immunostimulatory effect between the prepared emulsions. On the other hand, evaluation of the antitumor effect of both emulsion showed similar effect (Figure 4). Because the difference in footpad swelling between the two emulsions was slight, we could not detect difference in the antitumor effect on lymph node metastasis in guinea pig model. These results indicate that DTH experiment is sensitive enough to evaluate BCG-CWS formulations immunostimulatory effect *in vivo*.

Our group previously reported that SMP-105 is a TLR2 ligand that prevents tumors growth through TLR2 (*9*). Accordingly, we compare in this study the antitumor effect of SMP-105 emulsion to that of three launched TLR agonists (Aldara, Picibanil, and Immunobladder) (Figure 5). Although Aldara and Picibanil were administrated in sufficient amount and adequate route as indicated in drug package insert, they showed limited antitumor effect (Figure 5). SMP-105 emulsion showed potent antitumor effect equivalent to that of Immunobladder at low doses. This finding indicates that SMP-105 has promising clinical antitumor use.

Overall, we showed that squalane can be appropriate base material for SMP-105 emulsion and produced two types of squalane-based formulations that can be easily made into oil-in-water emulsions at the time of administration. The results of the prepared emulsions oil droplet distribution and DTH experiment suggest that small micrometer droplet (about 2-3 μm) can induce more potent immune reaction than large micrometer droplet (10 μm or larger). It is believed that small micrometer droplet is easily engulfed by phagocytes. This study is first to show that oil droplet size of few micrometer is optimal for SMP-105 microemulsion with squalane. We also showed for the first time that SMP-105 has more potent antitumor effect than launched TLR agonists in guinea pig metastasis model. These findings indicate that SMP-105 is a promising candidate for clinical investigation.

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Commentary

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Animal welfare and use of silkworm as a model animal

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ABSTRACT: Sacrificing model animals is required for developing effective drugs before being used in human beings. In Japan today, at least 4,210,000 mice and other mammals are sacrificed to a total of 6,140,000 per year for the purpose of medical studies. All the animals treated in Japan, including test animals, are managed under control of "Act on Welfare and Management of Animals". Under the principle of this Act, no person shall kill, injure, or inflict cruelty on animals without due cause. "Animal" addressed in the Act can be defined as a "vertebrate animal". If we can make use of invertebrate animals in testing instead of vertebrate ones, that would be a remarkable solution for the issue of animal welfare. Furthermore, there are numerous advantages of using invertebrate animal models: less space and small equipment are enough for taking care of a large number of animals and thus are cost-effective, they can be easily handled, and many biological processes and genes are conserved between mammals and invertebrates. Today, many invertebrates have been used as animal models, but silkworms have many beneficial traits compared to mammals as well as other insects. In a Genome Pharmaceutical Institute's study, we were able to achieve a lot making use of silkworms as model animals. We would like to suggest that pharmaceutical companies and institutes consider the use of the silkworm as a model animal which is efficacious both for financial value by cost cutting and ethical aspects in animals' welfare.

Keywords: Ethical issue, alternatives, developing drugs, medical studies, 3R, test animals, cost

It is said to be unavoidable to sacrifice model animals for developing effective drugs (*1*) before being used in human beings. However, use of mammalian animals has not been convenient due to high costs, long breeding times, and large and sophisticated space requirements. Another major limiting factor is ethical issues associated with the use of mammalian models (*2*). Various alternatives are being developed such as replacing mammals with cultured cells, but the effect of those is still limited (*3*). Therefore in Japan today, at least 4,210,000 mice and other mammals such as rats, guinea pigs, rabbits, dogs, cats, and monkeys are sacrificed to a total of 6,140,000 per year for the purpose of Medical studies (*4*).

All the animals treated in Japan, including test animals, are managed under control of "Act on Welfare and Management of Animals" enacted in 1973. The fundamental principle of this law is provided as "In light of the fact that animals are living beings, no person shall kill, injure, or inflict cruelty on animals without due cause, and every person shall treat animals properly by taking into account their natural habits and giving consideration to the symbiosis between humans and animals." (Article 2). This principle indicates that inflicting animals more pain than for inevitable tests is forbidden, even in necessary animal testing (Article 44) (*5*). It is required to minimize both numbers of test animals and the pain given to them. This principle has been basic and traditional in Western culture, and was already proposed in 1959, as the "3R" principle by W. M. Russell and by R. L. Burch in the UK (*6*). The three Rs are: "Replacement" – replacing the way of testing without test animals, "Reduction" – reducing the number of them, and "Refinement" – minimizing the quality and the intensity of pain given to animals to the least amount. "Animal" addressed in the principle can be defined as a "vertebrate animal" (*7*). In Japan, this principle was reflected in amendment of "Act on Welfare and Management of Animals", Article 44 in 2005.

If we can make use of invertebrate animals in testing instead of vertebrate ones, that would be a remarkable solution for the issue of animal welfare. There are numerous advantages of using invertebrate animal models: less space and small equipment are enough for taking care of a large number of animals and thus are

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cost-effective, they can be easily handled, there are less ethical issues surrounding their use, and many biological processes and genes are conserved between mammals and invertebrates. Many invertebrates have been used as animal models: fruit fly *Drosophila melanogaster* (*8,9*), grasshopper *Romalea microptera* (*10*), wax moth larva *Galleria mellonella* (*11*), honey bee *Apis mellifera* (*12*), and silkworm larva *Bomboyx mori* (*13*). Silkworms have many beneficial traits compared to mammals as well as other insects. Silkworms are not only easy for injection experiments but also they can be injected either through the intrahemolymph route that corresponds to intravenous in humans or the intramidgut route that corresponds to oral in humans. Figure 1 shows the easy ways of injecting silkworms through these routes. Unlike *D. melanogaster*, the silkworm has a large enough body size to carry out experiments for accurate dose administration and organ isolation. While injection into *Drosophila* requires special techniques and even though female flies are larger, they however have a hard outer surface which makes them difficult for injection. Unlike

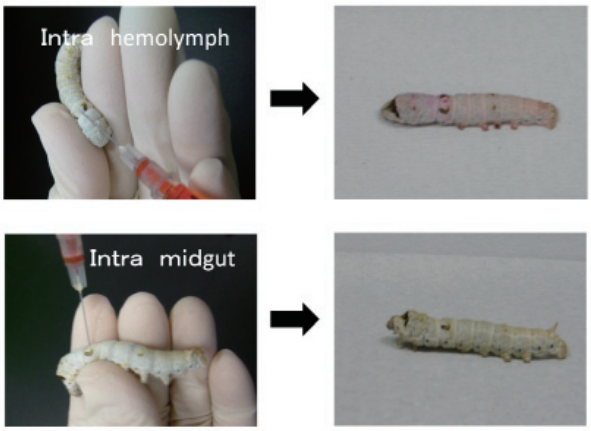

Figure 1. Injection into hemolymph and midgut. When injection angle is shallow, red ink is injected into blood (**Upper**). On the other hand, when injection angle is deep, it is injected into midgut and silkworm is not stained (**Lower**).

R. microptera and *G. mellonella*, the silkworm has an established method of breeding as it has been used for silk for over five thousand years. Unlike honey-bees, silkworms do not bite and even the adult moth cannot fly. The larvae do not have any sharp hair or horns that sting, so they are not harmful and do not require special techniques and caution for use. Moreover, the locomotion of silkworm larvae is slow which ensures that it cannot escape away from the laboratory setting easily which minimizes the risk of biological hazards. They can be fed an artificial diet that is easily available and can be bred all year round so that there is no shortage of larvae for experiments. The established rearing method allows the same kind of larval breeds which is very important for reproducible results. Larvae molt four times, it is easy to separate each instar larvae and they are stable having little individual genetic differences that give uniformity in research results. The whole genome is now known which allows for genetic manipulations and there are already many ongoing research projects that utilize transgenic and genetically modified silkworms.

Different models of the silkworm have already been established: bacterial infection model (*13,14*), baculoviral infection model (*15*), model to test innate immunity (*16-18*), diabetic model (*19*), bacterial virulence model (*20-22*), model to test pharmacokinetics (*23*), and model to test toxicity and metabolism (*24*). Most of these research projects have proven the correlation between results in mammals and silkworms. Table 1 distinguishes various features of the silkworm with other insects.

In the Genome Pharmaceutical Institute's study, we were able to achieve a lot making use of silkworms as model animals. Not only did we discover new effective chemicals including a new antibiotic "Kaikosin", but also we reduced the number of mice (small mammals in standards of test animals' size) and that helps towards making a solution for problems in animals' welfare, and cutting costs of testing. We are using silkworms instead of mice in the initial screening and testing in

Table 1. Comparision of silkworm with other insects

Items	Silkworm	Drosophila	Honey bee	Waxmoth	Grass hopper
Size	40-60 mm	1-3 mm	15-17 mm	30-40 mm	60-80 mm
Breeding method	Well established (> 5000 years)	Well established	Well established	Established	Established
Locomotion	Larva: slow, Adult: cannot fly	Flies	Flies	Larva: faster than silkworm, Adult: flies	Jumps, flies
Special handling technique	Not required	Required	Required	Not required	Required
Chance of biohazard	Less	Higher	Higher	Higher than silkworm, less than others	Higher
Injection technique	Easier, anyone can learn within couple of hours	Difficult, requires skilled personnel	--	Easier	--
Isolation of organs	Easier	Difficult ,not always possible	Easier	Easier	Easier
Route of administration/ Accuracy of administrated dosage	Oral, injection to dorsal surface: intrahemolymph, intramidgut/ accurate in case of injection	Oral, injection to dorsal surface, not accurate	--	Oral, topical, injection to ventral surface/ accurate in case of injection	--
Diseases models	Many	Many	--	Few	Few

Table 2. Experimental models used in screening antimicrobial agents from 10,000 chemical compounds and 15,000 natural compounds

Experimental models	10,000 chemical compounds	15,000 natural compounds
Our testing method	100 mice + 30,000 silkworms	150 mice + 45,000 silkworms
General method	30,000 mice	45,000 mice
'Reduction' effectiveness of mice	30,000 → 100 mice	45,000 → 150 mice

our company which is said to be a 'Replacement'. We used silkworms for screening chemical compounds as well as natural compounds. In screening for a curative effect with 10,000 chemical compounds' samples and 15,000 samples of products from soil bacteria (natural compounds) with antimicrobial activity, the number of mice used as test animals in our testing method is obviously less than that in the general method which here refers to tests in a mouse model directly after *in-vitro* analysis. We used silkworms after *in-vitro* analysis and before testing in the mouse model, and thus we could decrease the number of mice used for the tests. This decrement in the number of test mice is the 'Reduction' effectiveness (Table 2).

As shown in Table 2, we have gained a remarkable result in reduction of the number of mice in testing, by replacing them with silkworms in the initial testing. Having these results, we have been suggesting to pharmaceutical companies and institutes to consider the use of our silkworm related technologies, which is efficacious, with both a financial value by cost cutting and ethical aspects in animals' welfare. If we can replace 10% of tests run using mice as test animals in Japan, that means at least 400,000 mice can be saved from testing per year.

We should be aware of the fact that in Europe, more strict regulation, Cosmetics Directive 76/768/EEC was enacted in 2003. Dr. Tsutomu Kurosawa, the president of the society refers to the directive as "One of the epochs of our activities was the 7th amendment of EU cosmetics Directives in 2003. This directive ordered the total abundance of animal experimentation for cosmetic development and trade." (*25*). Based on this regulation, animal testing is gradually banned for developing materials and products for cosmetics, and products, subject to regulation of this Directive are already banned for sales in the EU. Some of the whole tests are still run, since a substitute way of testing without use of animals cannot be found, and it will take some more years for total abolishment. However, it won't be long before the day cosmetics produced using animal testing will be banned from sales in the EU. This is the global trend, and in the near future, animal usage alternatives or use of creatures, not controlled by the concept of animal welfare will be more valuable. With these issues, the silkworm is thus an ideal living creature for testing and can be counted on as a reliable test animal having great potential without animal welfare regulations.

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