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Naturally occurring enzyme inhibitors and their pharmaceutical applications*

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Abstract: Enzyme inhibitors play a significant role in the drug discovery process. For instance, acetylcholinesterase (AChE) inhibitors have applications in curing Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis, and Parkinson's disease. Glutathione *S*-transferase (GST) inhibitors have applications as adjuvants to overcome anticancer and antiparasitic drug resistance problems. Compounds inhibiting the activity of α-glucosidase are used to treat type 2 diabetes mellitus and obesity problems. This article describes the identification of natural products exhibiting AChE, GST, and α-glucosidase inhibitory activities from medicinally important plants. Additionally, structure–activity relationship (SAR) studies of these newly discovered enzyme inhibitors are also discussed.

Keywords: acetylcholinesterase; antibacterial activity; *Artocarpus nobilis*; *Barleria prionitis*; *Buxus hyrcana*; *B. natalensis*; α-glucosidase; glutathione *S*-transferase.

INTRODUCTION

A growing number of compounds with promising biomedical activity are supplied by natural product chemistry. Recent estimates indicate that approximately 50 % of commercially available antitumor and anti-infective agents are of natural products origin, and 25 % of these pharmaceuticals are of plant origin [1,2]. The discovery of combinatorial chemistry two decades ago was thought to be a cost-effective method for providing lead compounds to the drug discovery process. Unfortunately, this branch of chemistry has failed to provide the structural diversity compared to natural product chemistry. Despite extensive research, combinatorial chemistry has yielded only one anticancer drug, sorafenib [3], while almost half of the 877 small molecules introduced as pharmaceuticals between 1991 and 2002 are of natural products origin [4,5].

A key aspect of drug discovery is the identification of small molecules with enzyme-inhibiting activities. Enzymes are essential to human life, mediating biochemical processes including metabolism, cellular signal transduction, cell cycling, and development. Malfunction in these biochemical systems often leads to disease, the root cause of which can often be traced to the dysfunction, overexpression, or hyperactivation of the enzymes involved [6]. An understanding of diseases at the molecular level has led to the discovery of effective enzyme inhibitors that are used in clinical practice. Two such inhibitors are the cholesterol-lowering agent lovastatin (mevinolin) and the acetylcholinesterase (AChE) inhibitor galanthamine. Lovastatin acts at a key step in cholesterol biosynthesis, inhibiting the enzyme (3*S*)-hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase and preventing catalysis of the reduc-

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tion of HMG-CoA to mevalonate [7,8]. Galanthamine is a potent AChE inhibitor used in the treatment of Alzheimer's disease (AD) [9]. Our research group is involved in discovering new lead molecules exhibiting anti-AChE, anti-glutathione *S*-transferase (GST), anti-α-glucosidase, antioxidant, antileishmanial, and antimicrobial activities from medicinally important plants [10–13]. In this article, the structures and structure–activity relationships (SAR) of natural products exhibiting potent anti-AChE, anti-GST, and anti-α-glucosidase activities are described.

ACETYLCHOLINESTERASE INHIBITORS

Acetylcholine is a neurotransmitter, present in the central and peripheral nervous system, which plays an important role in signal transduction across synapses. AChE hydrolyzes acetylcholine into choline and acetic acid, thereby deactivating this function [14]. The consequent deficiency of acetylcholine in the nervous system is a predisposing factor in numerous neurological problems including AD. Accordingly, enhancement of acetylcholine levels in the brain is considered to be one of the most effective approaches to treat AD [14,15]. This can be accomplished by using potent AChE inhibitors. AChE inhibitors also play a role in preventing pro-aggregating activity of AChE leading to the deposition of β-amyloid, another cause of AD [16]. Four AChE inhibitors—tacrine, donepezil, galanthamine and rivastigmine—are approved by the U.S. Food and Drug Administration (FDA) to be used in clinics [16]. These compounds have limited effectiveness and a number of side effects [17,18]. For example, tacrine exhibits hepatotoxic lability and rivastigmine has a short half-life. AChE inhibitors also have applications in treating senile dementia, ataxia, myasthenia gravis, and Parkinson's disease [19]. In our continuing effort to discover new AChE inhibitors, our phytochemical investigation of *Buxus hyrcana*, collected from Iran, yielded two novel steroidal alkaloids, O^6-buxafurandiene **1** and 7-deoxy-O^6-buxafurandiene **2** exhibiting anti-AChE activity with IC_{50} values of 17.0 and 13.0 μM, respectively [20]. These studies prompted us to collect *B. natalesis* from South Africa based on the ethnomedicinal use of this plant to enhance memory in elderly people by the local traditional healers. The crude methanolic extract of this plant exhibited anti-AChE activity with an IC_{50} value of 28 μg/ml in our bioassay. AChE-inhibition directed phytochemical studies on this extract yielded two new natural products, O^2-natafuranamine **3**, O^{10}-natafuranamine **4** along with two known alkaloids, buxafuranamide **5**, and buxalongifolamidine **6** displaying anti-AChE activity with IC_{50} values of 3.0, 8.5, 14.0, and 30.2 μM, respectively [21]. The bioactivity of compound **3** was nearly identical to huperzine (IC_{50} = 2.0 μM), a standard AChE inhibitor used to treat AD. Compounds **1**, **2**, **4**, and **5** were nearly equally potent in AChE inhibition assay, suggesting the bioactivity of these compounds might be due to the presence of a THF ring incorporated in their structures. The structural analysis of these compounds further indicated that the location of an ether linkage in these compounds does not play any role in enzyme inhibition activity, as **1**, **2**, and **5** contain ether linkage between C-31 and C-6, while **4** has an ether linkage between C-31 and C-10. Compound **3** has an ether linkage between C-31 and C-2 and an epoxy functionality at C-1/C-10. The higher potency of this compound was assumed to be due to the presence of these two functionalities. Structures of **1–6** are shown in Fig. 1.

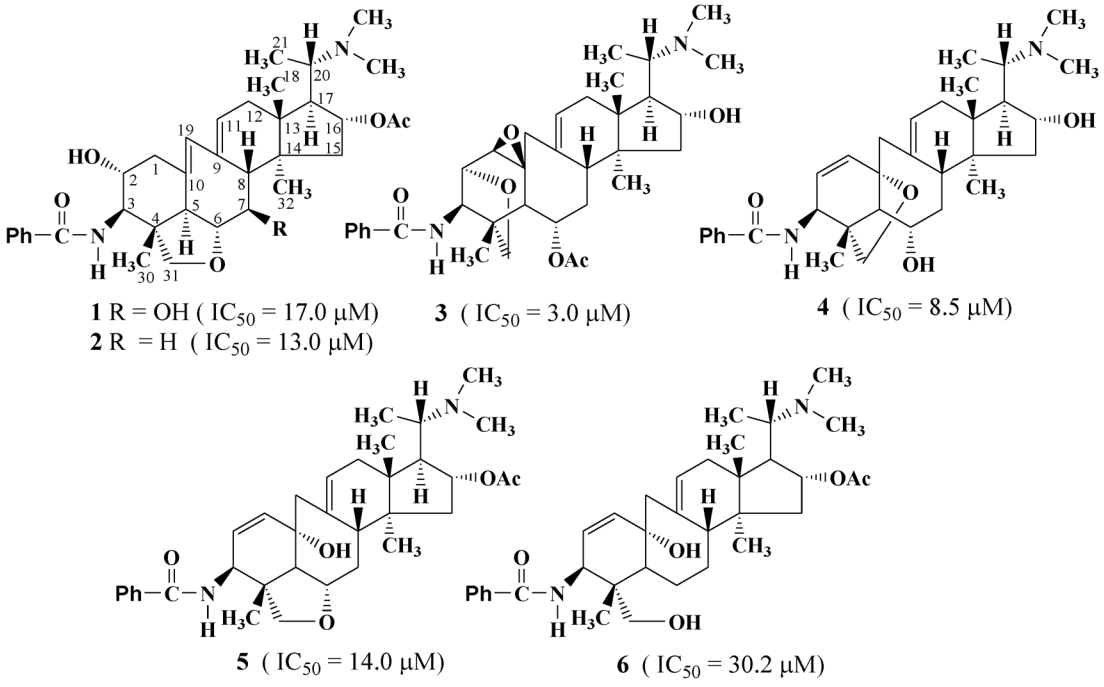


Fig. 1 Structures of compounds **1–6** and their IC_{50} values (concentration required to inhibit 50 % activity of enzyme) in AChE inhibition assay.

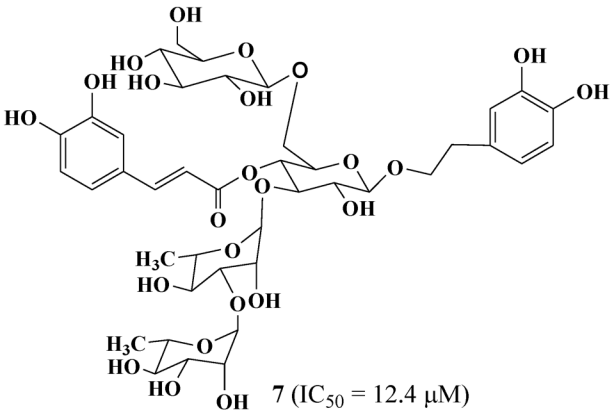
GLUTATHIONE *S*-TRANSFERASE INHIBITORS

Presently, a lot of research is underway to explain the mechanisms of acquired drug resistance during the treatment of cancer and parasitic diseases. The cystosolic detoxification enzyme, GST (E.C. 2.1.5.18), has been suggested to play an active role in this process [22]. GSTs are phase II detoxification isozymes that catalyze the reaction of various exogenous and endogenous electrophilic substances with glutathione to make adduct. Glutathione adducts are soluble in water and therefore can easily be excreted from the body [23]. Anticancer drugs with electrophilic centers can easily form this adduct in the presence of GST and can be excreted from the body, thus lowering the efficiency of these chemotherapeutic agents. In humans, GSTs exist in the form of various dimerized isoenzyme classes: α (A), μ (M), ω, π (P), θ (T), ζ (Z), and σ classes. Their existence in different forms has provided broad substrate specificities promoting detoxification of many toxic substances. Overexpression of GST in various human cancers was discovered compared to the normal tissues [24]. A 2-fold increase in GST activity has been reported in the literature in lymphocytes obtained from chronic lymphocytic leukemia (CLL) patients, resistant to chlorambucil when compared with untreated CLL patients. The effectiveness of cancer chemotherapeutic agents might be improved by the use of GST inhibitors as adjuvant during cancer chemotherapy. Toward this end, we screened several medicinally important plants in GST inhibition assay and discovered that the crude methanolic extracts of *Barleria prionitis*, *Nauclea latifolia*, and *Artocarpus nobilis* exhibited anti-GST activity with IC_{50} values of 160.0, 10.5, and 125 μg/ml, respectively. Phytochemical studies on *Barleria prionitis* yielded a new natural product, barlerinoside **7**, exhibiting GST inhibitory activity with an IC_{50} value of 12.4 μM. This bioactivity is more or less comparable with the GST inhibitory activity of a positive control, ethacrynic acid, a substrate GST inhibitor (IC_{50} = 16.5 μM). Compound **7** has also shown free radical scavenging activity with an IC_{50} value of 0.42 μg/ml [25]. Our chemical studies on the crude ethanolic extract of *Nauclea latifolia* yielded five known compounds, strictosamide **8**, naucleamides A **9**, naucleamide F **10**, quinovic acid-

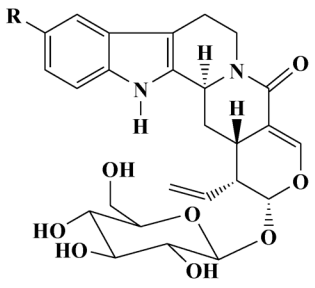
3-*O*-β-rhamnosylpyranoside **11**, and quinovic acid 3-*O*-β-fucosylpyranoside **12**. Compounds **8–12** showed GST inhibitory activity with IC_{50} values of 20.3, 37.3, 23.6, 143.8, and 53.5, respectively. Compound **8** showed significant anti-GST property and was isolated in a large quantity. It was, therefore, decided to carry out microbial reactions on this compound to prepare analogues and to evaluate them for GST inhibitory activity in order to study their SAR. To achieve this goal, we screened five different fungi, namely, *Mucor plumbeus* (ATCC 4740), *Cunninghamella blakesleeana* (ATCC 9245), *C. echinulata* (ATCC 9244), *Curvularia lunata* (ATCC 12017), *Rhizopus circinans* (ATCC 1225), and *Aspergillus niger* (ATCC 1004) for their capability to metabolize compound **8**. During these biotransformation experiments, we discovered that *C. blakesleeana* and *R. circinans* metabolized compound **8** into 10-hydroxystrictosamide (**13**), 10-β-glucosyloxyvincoside lactam (**14**), and 16,17-dihydro-10-β-glucosyloxyvincoside lactam (**15**). *C. blakesleeana* is reported to perform hydroxylation reactions on aromatic compounds while *R. circinans* was discovered for the first time to perform this reaction [26]. In order to determine the sequence for the formation of metabolites **13–15**, the time-dependent biotransformation experiments were also performed. These experiments were carried out by incubating compound **13** in the liquid culture of *R. circinans*; this afforded compounds **14** and **15**. We incubated compound **14** in the liquid culture of this fungus to get compound **15**. These results indicated that *R. circinans* initially performed microbial hydroxylation at C-10 of compound **8** to yield compound **13**. This metabolite further underwent glycosylation, followed by reduction of the $\Delta^{16–17}$ double bond to give compounds **14** and **15**, respectively.

Compounds **13–15** were also evaluated for anti-GST assay and found to exhibit anti-GST activity with IC_{50} values of 18.6, 12.3, and 46.6 μM, respectively [27]. Compounds **13** and **14** were found to be more potent compared to **8** (parent compound), and this might be possibly due to the introduction of polar groups that may have increased their solubility in water. Structures of compounds **7–15** are shown in Fig. 2.

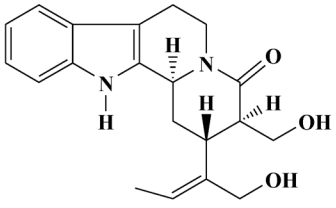
A detailed GST inhibition-directed chemical analysis of the ethanolic extract of *Artocarpus nobilis* Thw. (Moraceae) resulted in the isolation of five known flavonoids, artonins E **16**, artobiloxanthone **17**, artoindonesianin U **18**, cyclocommunol **19**, and multiflorins A **20**. Compounds **16–20** were significantly active in our GST inhibition assay with IC_{50} values of 2.0, 1.0, 6.0, 3.0, and 4.0 μM, respectively [28]. These bioactivity data indicated that compounds **16–20** are more potent GST inhibitors than ethacrynic acid. The structures of compounds **7**, **8**, **10**, **13**, **14**, and **16–20** all feature α,β-unsaturated carbonyl functionality, which suggests that this may constitute a pharmacophore for this expression of bioactivity. This was further confirmed by a significant decrease in the bioactivity of compound **15** (IC_{50} = 46.6 μM), in which a double bond adjacent to the carbonyl group was reduced by microbial reaction. The α,β-unsaturated carbonyl group would lead to the formation of a glutathione adduct of these compounds through Michael addition to inhibit the activity of GST [29]. Structures of compounds **16–20** are shown in Fig. 3.



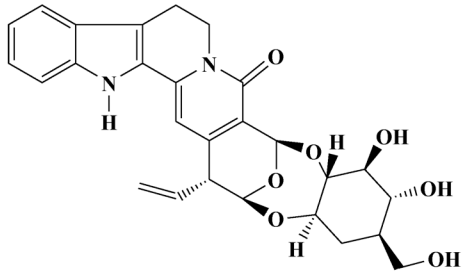
7 (IC_{50} = 12.4 μM)



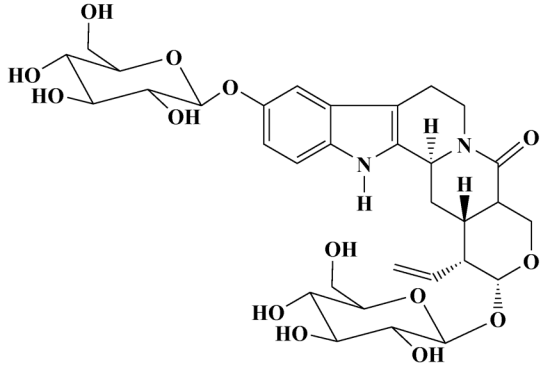
8 R = H (IC_{50} = 20.3 μM)
13 R = OH (IC_{50} = 18.6 μM)
14 R = β-D-glucose (IC_{50} = 12.3 μM)



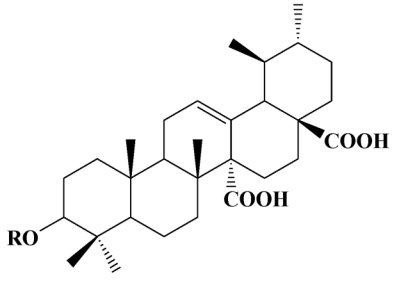
9 (IC_{50} = 37.2 μM)



10 (IC_{50} = 23.6 μM)



15 (IC_{50} = 46.6 μM)



11 R = α-Rhamnose (IC_{50} = 143.8 μM)
12 R = β-Fucose (IC_{50} = 53.5 μM)

Fig. 2 Structures of compounds **7–15** and their IC_{50} values in anti-GST assay.

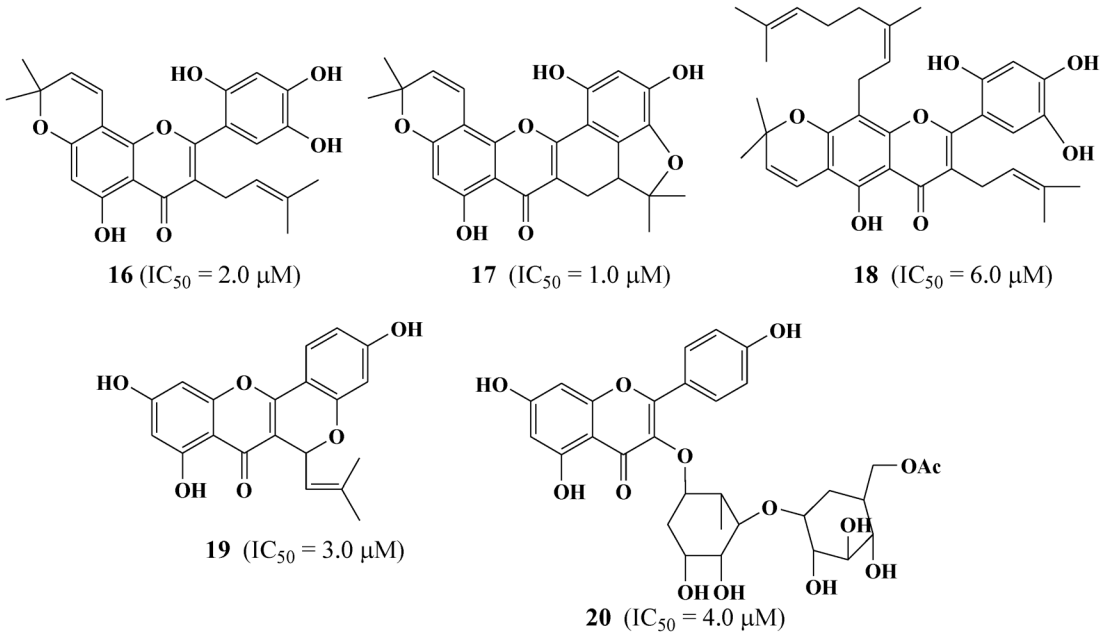


Fig. 3 Structures of compounds **16–20** and their IC_{50} values in GST inhibition assay.

α-GLUCOSIDASE INHIBITORS

α-Glucosidase is a membrane-bound enzyme that lies on the intestinal cells and catalyzes the final step of carbohydrate digestion to liberate free glucose, causing postprandial hyperglycemia [30]. This causes type 2 diabetes mellitus and affects approximately 2115 million people worldwide. The potent α-glucosidase inhibitors can be used to overcome this problem and to treat obese patient [31]. These compounds are also useful as antiviral, antimetastatic, immunomodulatory agents [32]. In this context, our recent phytochemical studies on the methanolic extract of *Drypetes gossweileri* afforded natural products displaying α-glucosidase inhibitory activity. These include *N*-β-D-glucopyranosyl-*p*-hydroxyphenylacetamide **21** (IC_{50} = 12.0 μM), *p*-hydroxyphenylacetic acid **22** (IC_{50} = 50.0 μM), *p*-hydroxyphenylacetonitrile **23** (IC_{50} = 48.0 μM), *p*-hydroxyacetophenone **24** (IC_{50} = 50.0 μM), 3,4,5-trimethoxyphenol **25** (IC_{50} = 56.0 μM), dolichandroside A **26** (IC_{50} = 20.0 μM), and β-amyrone **27** (IC_{50} = 25.0 μM) [33]. Compound **21** was found to be more potent compared to the rest of the isolates, and represented the first example of the plant natural product containing *N*-glucose moiety incorporated in its structure. Acidic hydrolysis of compound **21** afforded aglycone **28**, which exhibited α-glucosidase inhibition activity with an IC_{50} value of 60.0 μM, suggesting the higher potency of **21** was due to the presence of a *N*-glucose moiety.

Compounds **21** and **27** also exhibited antifungal activity against *Candida albicans* with minimum inhibitory concentration (MIC) of 8.0 μg/ml. Compound **28** was weakly active in this bioassay with an MIC value of 32 μg/ml, again indicating the higher potency of compound **21** was due to the presence of a *N*-glucose moiety in it. In an attempt to study the SAR of compound **27**, it was reacted with *m*-chloroperbenzoic acid to afford **29** and **30**. Both of these compounds were further treated with 20 % ammonium hydroxide solution to afford **31** and **32**, respectively. Compounds **29** (MIC = 4.0 μg/ml), **30** (MIC = 8.0 μg/ml), **31** (MIC = ≤2.0 μg/ml), and **32** (MIC = 9.0 μg/ml) exhibited antifungal activity. Compounds **29** (IC_{50} = 4.0 μM), **30** (IC_{50} = 10.0 μM), **31** (IC_{50} = 1.0 μM), and **32** (IC_{50} = 10.0 μM) also showed anti-α-glucosidase activity. These bioactivity data of compounds **29–32** suggested that the presence of a β-oriented C-12/C-13 epoxy functionality in **29** improve its bioactivity. It was further

observed that antifungal and α-glucosidase inhibition activities of compound **31** was significantly improved by the introduction of C-12α/NH_2 and C-13β/OH groups as these groups might be playing a role in binding with their target receptors through hydrogen bonding. These studies suggest that it is worthwhile to study the SAR on moderately bioactive natural products to improve their bioactivities. Structures of compounds **21–32** are presented in Fig. 4.

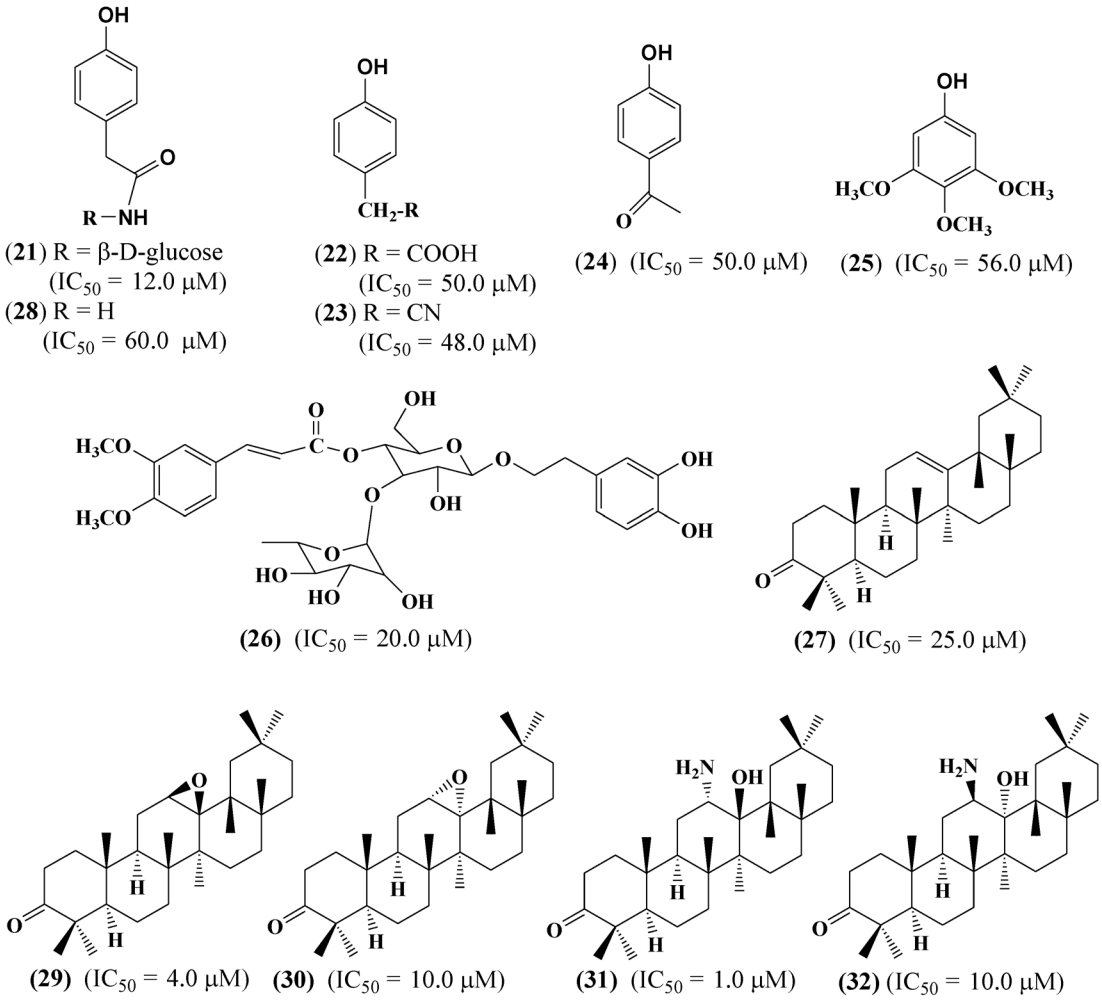


Fig. 4 Structures of compounds **21–32** and their IC_{50} values in anti-α-glucosidase assay.

CONCLUSION

In summary, our phytochemical studies on medicinally important plants have resulted in the identification of lead bioactive compounds exhibiting AChE, GST, and α-glucosidase inhibitory activities. For instance, compound **3** is identified as a potent AChE inhibitor while compounds **7**, **8**, and **16–20** have shown their potential as GST inhibitors whereas synthetic compound **31** exhibited the potent α-glucosidase inhibition and antifungal activities. The bioactivity data of **7**, **8**, and **13–20** suggested that an α,β-unsaturated carbonyl group may be acting as a pharmacophore the expression of GST inhibitory activity. Furthermore, compounds **16–20** were found to be more potent in vitro GST inhibitors than the

currently used GST inhibitor, ethacrynic acid. These compounds need to be screened for in vivo GST inhibition activity. SAR studies on compound **27** warrant SAR studies on moderately active natural products to improve their bioactivities.

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