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学位授与の日付	平成２２年３月３１日
論　文　名	Screening of edible plants for urease inhibitors and chemical Structures of the inhibitors （食用植物を対象としたウレアーゼ阻害物質の検索とその化学構造）
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論文要旨

A variety of ureases are found in bacteria, fungi, higher plants, and in soil as soil enzymes. Medically bacterial ureases are important virulence factors implicated in the pathogenesis of many clinical conditions such as pyelonephritis, hepatic coma, peptic ulceration, and the formation of infection-induced urinary stones. In agriculture, high urease activity causes significant environmental and economic problems through ammonia toxicity which induces plant damage. Urease is a virulence factor of *Helicobacter pylori* which is a gram negative bacterium causing gastric ulcers and stomach cancer. Thus urease inhibitors have recently attracted much attention as new potential antiulcer drugs, and many urease inhibitors have been described in the last few decades, however some of these compounds were prohibited for in vivo use because of their toxicity or instability. On the other hand, more attention has been focused on exploring novel biological properties of phytochemicals isolated from edible plants. Thus edible plants may be very useful resources for safe urease control.

In this study, the methanolic extracts of 125 samples of edible plants, foodstuffs, and seaweeds were screened for their inhibitory activity against Jack bean urease. As a result, 5 samples (mung bean, pineapple, cinnamon, avocado peel, and guava) showed high inhibitory activity in the range of 71%-100%, 10 samples (apple, red onion, pepper, black gram, licorice, cummin, sudachi peel, lemon peel, water melon, and coffee beans) the moderate activity in the range of 31%-70%, and 3 samples (cherry, satsuma, and bitter orange) low activity in the range of 20%-30%. The bulbs of *Allium cepa* and leaves of *Psidium guajava* were selected for further investigation of their urease inhibitory constituents.

Assay of urease inhibition was carried out using Jack bean urease which has been widely used as an enzyme for inhibitor screening. Reaction mixtures composing of 0.2 ml (0.47U) of urease solution and 1.2 ml of buffer (60 mM MES buffer, pH 6.0) were incubated with 0.1 ml of a sample solution at 37°C for 5 min in test tubes. After pre-incubation, 0.5 ml (0.05 mM) of urea was added to the reaction mixture and incubated for 20 min. Urease activity was determined by measuring ammonia production using the indophenol method described by Weatherburn.

Freshly collected peel from red onion (*A. cepa*) (2 Kg) was exposed to methanol, and the aqueous concentrate obtained was extracted with ethyl acetate. The active ethyl acetate extract was purified using a silica gel C-200 column with various solvent systems to afford active compound **1** (18 mg). The molecular formula of compound **1** was established to be $C_{21}H_{20}O_{12}$ by FAB-MS (*m*/z 465 $[M+1]^+$) together with ^{1}H-NMR (400 MHz, CD_3OD) and ^{13}C-NMR (100 MHz, CD_3OD) data. The ^{1}H- and ^{13}C-NMR spectra of compound **1** showed signals of two aromatic protons with meta coupling at ä$_H$ 6.11 (H-6, d. *J* = 1.6 Hz; ä$_C$ 100.32), ä$_H$ 6.31 (H-8, d. *J* = 1.6 Hz; ä$_C$ 95.46), which corresponded to a tetra-substituted benzene ring. Another 1,3,4-trisubstituted benzene ring was shown by the signals at ä$_H$ 7.68 (H-2`, d. *J* = 2.4 Hz; ä$_C$ 117.48), ä$_H$ 7.63 (H-6`, dd. *J* = 8.4, 2.4 Hz; ä$_C$ 122.24), and ä$_H$ 7.23 (H-5`, d. *J* = 8.4 Hz; ä$_C$ 118.6). These data together with ^{13}C-NMR signals attributed to a ketone carbonyl C-4 (ä$_C$ 178.39) and two oxygenated olefinic carbons C-2 (ä$_C$ 149.05) and C-3 (ä$_C$ 138.9) strongly indicated that compound **1** was a flavonol. The flavonol was confirmed to be quercetin by comparing its signals with those of standard reference and previously reported. An anomeric proton signal at ä$_H$ 4.87 (H-1``, d. *J*=7.2 Hz), signals of four oxygenated methine carbons at ä$_C$ 75.82, 78.54, 72.31 and 79.36 and a signal of one oxygenated methylene at ä$_C$ 63.43 indicated that **1** was quercetin β-glucoside. The connectivity of sugar unit was established from HMBC spectrum showing a correlation between anomeric proton H-1`` (ä$_H$ 4.87) and C-4` of quercetin (ä$_C$ 148.83). On the basis of all spectroscopic data and comparison of MS, $^1$H-NMR, and $^{13}$C-NMR data with those previously reported, compound **1** was identified as quercetin 4`-*O*-â-D-glucopyranoside.

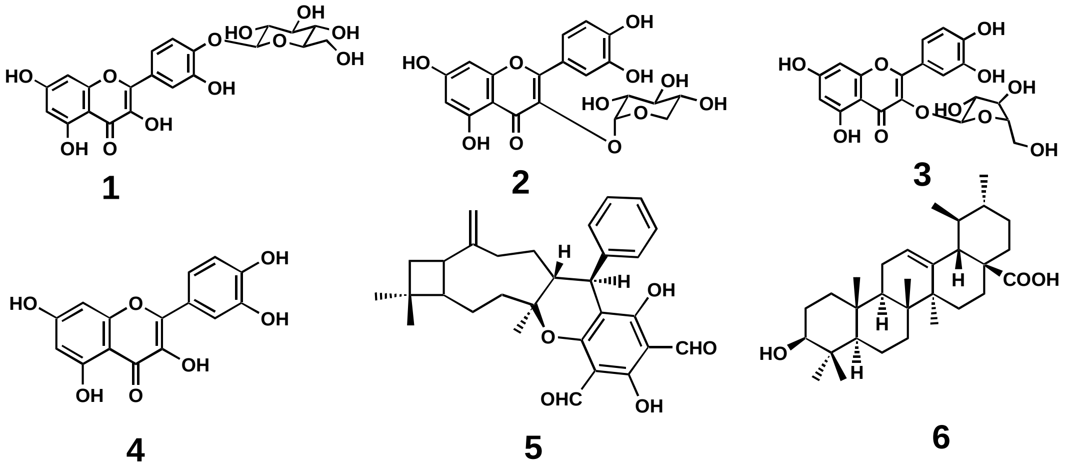
Dried leaves of *P. guajava* (2 Kg) were soaked in methanol, and the aqueous concentrate obtained was extracted with ethyl acetate. Repeated column chromatography of the active ethyl acetate extract on silica gel, ODS and sephadex LH-20 led to the isolation of compounds **2** (3 mg), **3** (8 mg), **4** (10 mg), **5** (5 mg), and **6** (3 mg). Compounds **2**, **3**, and **4** were identified as quercetin 3-*O*-α-L-arabinopyranoside (guaijaverin), quercetin 3-*O*-α-L-arabinofuranoside (avicularin), and quercetin, respectively. Compounds **5** and **6** were identified as guajadial and ursolic acid, respectively, by comparing their MS, ^{1}H-NMR, and ^{13}C-NMR data with those previously reported.

Inhibitory activity of the compounds **1**, **2**, **3**, and **4** together with quercetin 3-*O*-α-L-rhamnopyranoside (quercitrin) (**7**), quercetin 3-*O*-â-D-glucopyranoside (isoquercitrin) (**8**) was evaluated using Jack bean urease. The IC_{50} values of **1**, **2**, **3**, **4**, **7**, and **8** were determined to be 190 µM, 120 µM, 140 µM, 80 µM, 200 µM, and 160µM, respectively. These data indicated that quercetin (**4**) showed the strongest inhibitory activity against Jack bean urease. The glycosilation of hydroxy groups decreased the inhibitory activity. Guaijaverin (**2**) and avicularin (**3**) were slightly more active than other glycosides, while quercetin 4`-*O*-β-D-glucopyranoside (**1**) showed less inhibition activity than compounds **2** and **3**, this result suggested that the glycosilation of 3-hydroxy group decreased the activity and that the glycosilation of 4`-hydroxy decreased the activity to more extend. This illustrated why quercetin 3-*O*-â-D-glucopyranoside (isoquercitrin) showed inhibitory activity more than compound **1**. Quercitrin showed inhibitory activity less than other glycosides due to its content of 6-deoxy sugar rhamnose, suggesting that free hydroxy groups of sugar are important for inhibitory activity. Conformation of the sugar could affect the inhibitory activity, for guaijaverin with arabinopyranose was more active than avicularin with arabinofuranose. Flavones such as kampferol, apigenin, luteolin and baicalein have been reported to exhibit inhibitory activity against urease, indicating that 5-and 7-hydroxy groups at A-ring of flavones play an important role in exhibiting the inhibitory activity.

Inhibitory activity of the compounds **1**, **2**, **3**, and **4** together with **7**, and **8** was also evaluated using *Lactobacillus fermentum* urease. *L. fermentum* urease was used as comparison because this enzyme exhibits the activity in the same pH range as *H. pylori* urease. Quercetin 4`-*O*-β-D-glucopyranoside (**1**) and avicularin (**3**) showed a moderate inhibitory activity at a concentration of 100 µM while other compounds showed little or no activity.

The inhibitory activity of the compounds **5** and **6** was evaluated at concentrations of 210 µM and 220 µM, respectively against both Jack bean and *L. fermentum* ureases. Guajadial (**5**) showed moderate inhibitory activity against both enzymes, while ursolic acid (**6**) showed a little activity. Urease inhibitory activity of compounds **1**, **2**, **3**, **5**, and **6** was reported for the first time in this study.

From the important results in this study, it can be suggested that the edible plants are important and promising natural resources for discovery of novel bioactive compounds such as natural urease inhibitors which may be considered as safe and powerful anti ulcer drugs.



Structures of compounds (1-6)

審査結果の要旨

ウレアーゼは尿素をアンモニアと二酸化炭素に加水分解する酵素であり，様々な細菌や菌類，植物など自然界に広く存在している。しかしながら，ウレアーゼに対する特異的な阻害剤は未だ見いだされていない。尿素を利用可能なアンモニアに転化するウレアーゼは植物にとって重要な酵素であるものの，土壌中または地表に生息する微生物のウレアーゼにより尿素があまりにも迅速に加水分解された場合，発生したアンモニアによる植物への傷害，pH 上昇による土壌のアルカリ化などの問題が引き起こされる。また，腸内細菌科に属する *Proteus mirabilis* やピロリ菌（*Helicobacter pylori*）が有するウレアーゼは人間の疾病にも深く関与していることが明らかにされてきている。したがって，新規ウレアーゼ阻害物質の発見は酵素への知見を深めるのみならず，農薬や医薬品としての幅広い活用が見込まれる。

本研究では日本産およびエジプト産の食用植物を中心にウレアーゼ阻害物質の検索を行った。まず，食用植物 125 種の可食部から得られたメタノール抽出物について，ナタマメ由来のウレアーゼに対する阻害活性を検索した。その結果，5 種の植物（リョクトウ，パイナップル，シナモン，アボガド，グアバ）に顕著な阻害活性が，また 10 種の植物（リンゴ，タマネギ，コショウ，ケツルアズキ，カンゾウ，クミン，スダチ，レモン，スイカ，コーヒー）に中程度の阻害活性が認められた。本研究では、阻害活性の

認められた植物の中からタマネギ（*Allium cepa*）の鱗茎およびグアバ（*Psidium guajava*）の葉に含まれる活性物質の化学構造の解明を行った。

タマネギ鱗茎（2 kg）のメタノール抽出物を酢酸エチルおよびブタノールで順次抽出した。阻害害活性の認められた酢酸エチル抽出画分を，各種カラムクロマトグラフィーを用いて精製し，活性物質 1（18 mg）を単離した。各種スペクトルデータの解析から，化合物 1 を quercetin 4’-*O*-β-D-glucopyranoside と同定した。

次に，エジプト産植物であるグアバ葉に含まれる活性物質の解明を行った。乾燥葉（2 kg）をメタノールで抽出し，濃縮後酢酸エチルで抽出した。得られた抽出物から酵素阻害活性を指標として活性物質の精製を行い，quercetin（10 mg）とともに 2 種の quercetin 配糖体 2（3 mg）および 3（8 mg）を単離した。各種スペクトルデータを解析し，化合物 2 および 3 をそれぞれ quercetin 3-*O*-α-L-arabinopyranoside（guaijaverin）および quercetin 3-*O*-α-L-arabinofuranoside（avicularin）と同定した。

単離同定された quercetin および quercetin 配糖体（1，2，3）とともに市販の quercetin 配糖体である quercetin 3-*O*-α-L-rhamnopyranoside（quercitrin，4）と quercetin 3-*O*-β-D-glucopyranoside（isoquercitrin，5）のウレアーゼ阻害活性を評価した。ウレアーゼの阻害剤として汎用される acetoxyhydroxamic acid の ID_{50} 値が 20 ・M であるのに対し、ナタマメ由来のウレアーゼに対する quercetin の ID_{50} 値は 80 ・M であり，配糖体 1 および 2，3，4、5 の値はそれぞれ 190 ・M および 120 ・M，140 ・M，200 ・M，160 ・M であった。すなわちアグリコンである quercetin が最も強い阻害活性を示し，配糖体化による活性の低下が認められた。一方，*Lactobacillus fermentum* 由来のウレアーゼに対する阻害活性を 100 ・M の濃度で調べたところ，acetoxyhydroxamic acid が 70％の阻害活性であるの対して，quercetin は 12％の阻害活性と低いものであった。 また，配糖体 1 および 3 はそれぞれ 53% および 48%の阻害活性を示し，その他の化合物は活性を示さなかった。

グアバ葉からのフラバノール類を精製する過程で阻害活性を示すテルペノイド類 6（5 mg）および 7（3 mg）を単離した。各種スペクトルデータを解析し，6 をメロテルペノイドである guajadial，7 をトリテルペノイドである ursolic acid と同定した。化合物 6 は 210 ・M の濃度でナタマメ由来のウレアーゼに対して 45％，*L. fermentum* 由来のウレアーゼに対して 48％ の阻害活性を示した。また，化合物 7 の両酵素に対する阻害活性はそれぞれ 30％ および 21％ であった。

以上のように，本邦産およびエジプト産食用植物からウレアーゼ阻害物質としてフラボノールである quercetin とその配糖体，さらにはテルペノイド類を単離・同定したことから，食用植物が有用天然物の供給源として多いに期待されることが示された。また，フラボノール配糖体およびテルペノイドに新たな生理活性を見いだした。これらの成果は天然物化学および食品化学の両分野に多大の貢献をするものと考えられ、最終試

験の結果と併せて，博士（応用生命科学）の学位を授与することを適当と認める。