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Isolation of β-sitosterol from *Hibiscus sabdariffa* L.

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ABSTRACT

The aqueous methanol extracts of *H. sabdariffa* inhibited the growth of 12-test plant species, cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.), timothy (*Phleum pratense* L.), Italian ryegrass (*Lolium multiflorum* Lam.), ryegrass (*Lolium rigidum* Gaud.), crabgrass (*Digitaria sanguinalis* L.), buckwheat (*Eriogonum compositum* Douglas ex Benth.), Chinese sprangletop (*Leptochloa chinensis* [L.] Nees.), jungle rice (*Echinochloa colona* [L.] Link.), barnyardgrass (*Echinochloa crus-galli* [L.] Beauv.) and sand fescue (*Festuca myuros* L.) in a dose-dependent manner. The aqueous methanol extract of *H. sabdariffa* was purified and an active substance was isolated and identified as β-sitosterol by ^{1}H- and ^{13}C-NMR analysis. The threshold of β-sitosterol for growth inhibition was 0.3 µM. The I_{50} values on cress and lettuce were 16.2 and 406.7 µM, respectively, whereas these values were 10.2 and 61.0 µM on timothy and Italian ryegrass. The endogenous concentration of β-sitosterol in *H. sabdariffa* was 32.7 µmol/kg.

Key words: Alfalfa, aqueous methanol extract, β-sitosterol, barnyardgrass, buckwheat, Chinese sprangletop, crabgrass, cress, Italian ryegrass, jungle rice, lettuce, ryegrass, sand fescue, timothy.

INTRODUCTION

Widespread use of synthetic herbicides has resulted in the emergence of herbicide-resistant weeds and environmental concerns about the safety of synthetic herbicides. Therefore alternative weed management systems using naturally occurring compounds with lower synthetic herbicide dependency are required (34,35). Allelopathy holds promise for the environmentally friendly weed management. Allelopathy is the ability of plants to inhibit the germination of other plants through the production of allelochemicals which may be present in any part of the plants, i.e. leaves, roots, fruits, stems, rhizomes and seeds, from where they are released to the soil through volatilization, root exudation, leaching and decomposition of plant residues (31). The importance of allelopathy in biological control of weeds and crop productivity has been recognized and various methods to measure the allelopathic effects have been suggested (16,36,37). Numerous plants possess allelopathic potential and efforts have been made to apply them for weed control.

Allelopathic effects of medicinal plant species are of special interest in recent years (18,22). Fujii *et al.* (13,14,15) evaluated the allelopathic potentials of 239 medicinal

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plants using the "Plant Box Method" and 223 species suppressed the tested plant growth, whereas 17 species enhanced lettuce radicle growth. Furthermore, a survey of 387 Japanese medicinal plants reported that large number of species with allelopathic potentials, whose toxicity varied within the same plant species growing in different habitats. Gilani *et al*. (17) also surveyed allelopathic potential of 81 Japanese medicinal plants to find out possible candidates as natural herbicides. Nazir *et al*. (28) evaluated allelopathic potential of 3 herbal species (*Rheum emodi, Saussaurea lappa* and *Potentilla fulgens*) against some traditional crops. Germination of all crops was reduced significantly by aqueous extracts of *S. lappa* and *P. fulgens*. Aziz and Fujii (3) examined allelopathic activities of 14 medicinal plant species grown in plain areas of Pakistan with semi-arid conditions on growth of lettuce (*Lactuca sativa*).

Hibiscus sabdariffa L. (family Malvaceae), called roselle or red sorrel in English, is widely grown in tropics and subtropics of both hemispheres and has become naturalized in many other areas (27). *H. sabdariffa* is an annual, erect, bushy, herbaceous sub-shrub that grows to 2.4 m in height. The thick, red and fleshy, cup-shaped calyces of the flower are consumed worldwide as a cold beverage and as a hot drink (sour tea). This plant contains many bioactive secondary metabolites to treat many diseases (high blood pressure, liver diseases and fever) and against inflammation (10,32,38). However to date, no information is available on the allelopathic compounds in *H. sabdariffa*. In this study, a phytotoxin was isolated from the methanol extracts of *H. sabdariffa*. The substance was characterized and its biological activities and endogenous concentrations of the substance were determined.

MATERIALS AND METHODS

I. Plant materials

Whole plants (leaves, stem and roots) of *Hibiscus sabdariffa* L. were collected from Chiang Mai province, Thailand, in August 2011. The plants were washed several times by tap water, dried under sunlight and then ground into powder. Cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.) and timothy (*Phleum retense* L.) were selected as test plants for bioassay because of their known seedling growth behavior. Italian ryegrass (*Lolium multiflorum* Lam.), ryegrass (*Lolium rigidum* Gaud.), crabgrass (*Digitaria sanguinalis* L.), buckwheat (*Eriogonum compositum* Douglas ex Benth.), Chinese sprangletop (*Leptochloa chinensis* [L.] Nees.), jungle rice (*Echinochloa colona* [L.] Link.), barnyardgrass (*Echinochloa crus-galli* [L.] Beauv.) and sand fescue (*Festuca myuros* L.) were selected as test plants for bioassay because these are common weeds and distributed in crop fields.

II. Extraction and bioassay

Plant powder (500 g) was extracted with 2 L of 80% (v/v) aqueous methanol for two days. The extract was filtered through one layer of filter paper (No. 2; Toyo Ltd., Japan), using a vacuum pump. The residue was extracted again with 2 L cold methanol for one day and filtered. The two filtered were combined and evaporated with a rotary evaporator at 40°C.

An aliquot of extract (final assay concentration was 10, 30, 100 and 300 mg dry weight equivalent extract/mL) was evaporated to dryness at 40°C *in vacuo* by rotary evaporator, dissolved in 3 mL methanol and added to a sheet of filter paper (No. 2) in a 2.8 cm Petri dish. The methanol was evaporated in a draft chamber, then the filter paper was moistened with 0.6 mL of 0.05% (v/v) aqueous solution of polyoxyethylenesorbitan monolaurate (Tween 20; Nacalai, Kyoto, Japan), which was used as surfactant and did not cause any toxic effects. Timothy, sand fescue and buckwheat were germinated in the darkness at 25°C for 48 h, crabgrass, barnyardgrass and jungle rice were germinated in the darkness at 25°C for 120 h, and Italian ryegrass, ryegrass and Chinese sprangletop were germinated in the darkness at 25°C for 72 h. Then, 10 seeds of cress, lettuce or alfalfa, or 10 germinated seeds of timothy, sand fescue, buckwheat, crabgrass, barnyardgrass, jungle rice, Italian ryegrass, ryegrass or Chinese sprangletop were arranged on the filter paper in Petri dishes. The aqueous solution of Tween 20 without the extract was used as control. The shoot and root lengths of seedlings were measured at 48 h after incubation in dark at 25°C. The bioassay was repeated thrice with 10 plants for each determination. The inhibition (%) was calculated as under:

$$\text{Inhibition (\%)} = [1- (\text{length of test plants} / \text{length of control plants})] \times 100.$$

III. Purification of active substance

Hibiscus sabdariffa L. (500 g dry weight) was extracted as described above and the extract was concentrated at 40°C *in vacuo* to produce aqueous residue. The aqueous residue was adjusted to pH 7.0 with 1 M phosphate buffer, partitioned three times against an equal volume of ethyl acetate. The aqueous residue was then divided into aqueous and ethyl acetate fraction (Fig. 1). The biological activity of fractions was determined using a cress bioassay as described above. The ethyl acetate fraction was evaporated to dryness and chromatographed on a column of silica gel (60 g, silica gel 60, 70–230 mesh; Merck), eluted stepwise with *n*-hexane containing increasing amounts of ethyl acetate (10% per step, v/v; 100 mL per step). The biological activity was determined in the fraction by elution in 70%-80% ethyl acetate in *n*-hexane and gave 80 mg residue. After evaporation, the residue was separated by a column of Sephadex LH-20 (100 g, Amersham Pharmacia Biotech, Buckinghamshire, UK), and eluted with 20, 40, 60 and 80% (v/v) aqueous methanol (100 mL per step) and methanol (200 mL). The active fractions were eluted by 40% aqueous methanol and evaporated to dryness. The residue was dissolved in 20% (v/v) aqueous methanol (2 mL) and loaded onto reverse phase C_{18} Sep-Pak cartridges (Waters). The cartridge was eluted with 20, 40, 60, 80% (v/v) aqueous methanol and methanol (15 mL per step). The active fraction was eluted by 40% aqueous methanol and evaporated to dryness. The residue of active fraction was finally purified by reverse-phase HPLC (10 mm i.d. × 50 cm, ODS AQ-325; YMC Ltd., Kyoto, Japan) eluted at a flow rate of 1.5 mL/min with 50% aqueous methanol, detected at 220 nm.

IV. Structural characterization of active substance

1D and 2D NMR spectra of the active substance were recorded on a JEOL ECA-600 spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C) at 25°C with tetramethylsilane (TMS) as an internal standard.

V. Bioassay of β-sitosterol

Growth inhibitory activity of β-sitosterol was determined by dose-response experiments. Filter paper was placed into Petri dish and test solution of β-sitosterol was added on it. Final concentrations of β-sitosterol were 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 or 100 μM. After the solvent evaporated, 10 seeds of cress or lettuce, or 10 germinated seeds of timothy or Italian ryegrass were arranged on the filter paper in the Petri dishes (Fig. 2). For control treatments, seedlings were placed into the filter paper moistened with the aqueous solution of Tween 20 without the solution of β-sitosterol.

Shoot and root lengths were measured after 48 h incubation in dark at 25°C. The percentage length of seedlings was then determined by reference to the length of control seedlings. The concentrations required for 50 % inhibition (defined as I_{50}) of the test plants in the assay was calculated from the regression equation of the concentration-response curves.

VI. Statistical analysis

All experimental treatments were replicated thrice and repeated twice. Treatments were prepared in a completely randomized design. Data were analyzed by SPSS version 16.0 using One-way ANOVA.

RESULTS

Phytotoxic activity of aqueous methanol extract of *H. sabdariffa*

Aqueous methanol extracts of *H. sabdariffa* at 10 mg dry weight concentration of stimulated the shoot and root growth of crabgrass and ryegrass, but inhibited the shoot and root growth of another test plant species (Table 1). The inhibitory effect increased with increasing concentrations of extracts. The extracts obtained from 100 mg dry weight of *H. sabdariffa* plants completely inhibited (100%) the shoot growth of cress, lettuce and alfalfa seedlings and shoot growth of timothy and crabgrass, Italian ryegrass, ryegrass, buckwheat, Chinese sprangletop, jungle rice, barnyardgrass or sand fescue were inhibited by 56.3, 73.9, 75.7, 58.5, 48.5, 80.4, 54.4, 86.4, and 81.1%, respectively. At concentration of 300 mg/mL, shoot growth of timothy, crabgrass, Italian ryegrass and sand fescue were completely inhibited. Comparing the concentration required for 50% inhibition (Table 2), barnyardgrass shoots were the most sensitive to the extracts follow by cress and alfalfa. The shoot growth of timothy was less sensitive to the extracts.

The extracts obtained from 100 mg dry weight of *H. sabdariffa* plants completely inhibited the root growth of cress, lettuce and alfalfa seedlings (100%) and root growth of timothy and crabgrass, Italian ryegrass, ryegrass, buckwheat, Chinese sprangletop, jungle rice, barnyardgrass or sand fescue were inhibited by 66.6, 73.7, 78.2, 64.5, 70.9, 84.6, 79.4, 89.2, and 90.7%, respectively (Table 1). At concentration of 300 mg/mL, the timothy, crabgrass, Italian ryegrass and sand fescue roots growth was completely inhibited. Comparing the concentration required for 50% inhibition, barnyardgrass roots were most sensitive to extract of *H. sabdariffa* followed by cress and alfalfa. Italian ryegrass roots were less sensitive to the extracts (Table 2).

Table 1. Inhibition (%) of aqueous methanol extracts of *H. sabdariffa* on the growth of test plants

Test plant species	Inhibition (%)							
	Shoot				Root			
	Concentration (g dry weight equivalent extract/mL)							
	0.01	0.03	0.1	0.3	0.01	0.03	0.1	0.3
Cress	38.7a	97.00b	100.0b	100.0b	53.0a	97.4b	100.0b	100.0b
Lettuce	4.4a	78.1b	100.0b	100.0b	29.67a	84.6b	100.0b	100.0b
Alfalfa	36.4a	96.9b	100.0b	100.0b	46.1a	95.0b	100.0b	100.0b
Timothy	3.6a	29.3a	56.3ab	100.0b	5.70a	33.3ab	66.6bc	100.0c
Crabgrass	-16.1a	21.6ab	73.9bc	100.0c	-20.8a	28.1ab	73.7bc	100.0c
Italian ryegrass	7.9a	37.9ab	75.7bc	100.0c	44.7a	58.4ab	78.2b	100.0c
Ryegrass	2.1a	39.3ab	58.5b	78.2b	-10.7a	27.2ab	64.5b	85.9b
Buckwheat	17.0a	39.3a	48.5ab	74.9b	32.6a	55.8ab	70.9b	83.9b
Chinese sprangletop	29.7a	55.0b	80.4c	92.4c	30.3a	56.1b	84.6c	95.6c
Jungle rice	12.0a	37.9b	54.4b	91.0 c	23.7a	63.5b	79.4bc	94.2c
Barnyardgrass	17.2a	69.3b	86.4c	96.1c	44.0a	81.9b	89.2b	97.1b
Sand fescue	13.1a	55.8b	81.1c	100.0c	24.9a	52.4b	90.7c	100.0c

Mean with same letters in a row is not significantly different at $P < 0.001$.

Table 2. I_{50} values of aqueous methanol extracts of *H. sabdariffa* for shoots and roots of test plants.

Test plant species	I_{50} (g dry weight equivalent extract/mL)	
	Shoot	Root
Cress	5.8	5.8
Lettuce	6.8	6.5
Alfalfa	5.8	5.8
Timothy	135.1	70.6
Crabgrass	39.3	26.7
Italian ryegrass	43.8	98.8
Ryegrass	16.8	28.7
Buckwheat	93.9	21.7
Chinese sprangletop	25.4	25.4
Jungle rice	124.3	7.8
Barnyardgrass	1.2	4.1
Sand fescue	15.4	26.7

The values were determined by a logistic regression analysis after bioassays

Purification of phytotoxic substance and biological activity

The active component in the aqueous methanol extract of *H. sabdariffa* was purified by bioassay-guided fractionation (Fig. 1). Two fractions of aqueous and ethyl acetate were separated from the aqueous methanol extract and their biological activities were determined. Both fractions suppressed the root and shoot growth of cress seedlings. The ethyl acetate fraction proved more inhibitory (Fig. 3). Thus, phytotoxic substances were isolated using the ethyl acetate fraction.

The ethyl acetate fraction was separated by columns of silica gel and the biological activity was found in the fractions eluted with 70%-80% ethyl acetate in

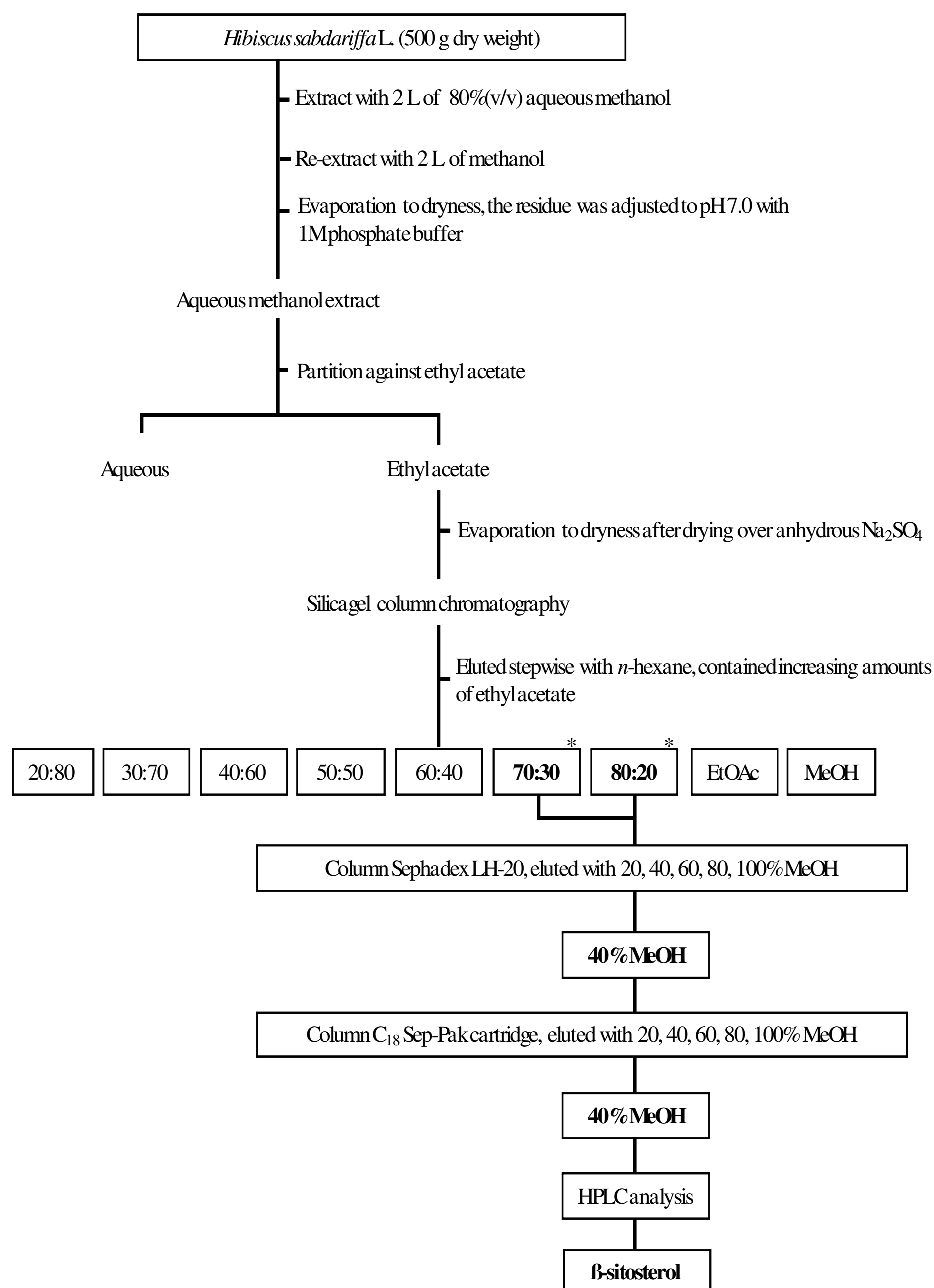

Figure 1. Procedure for isolation of an active compound from *H. sabdariffa*, * Active fractions

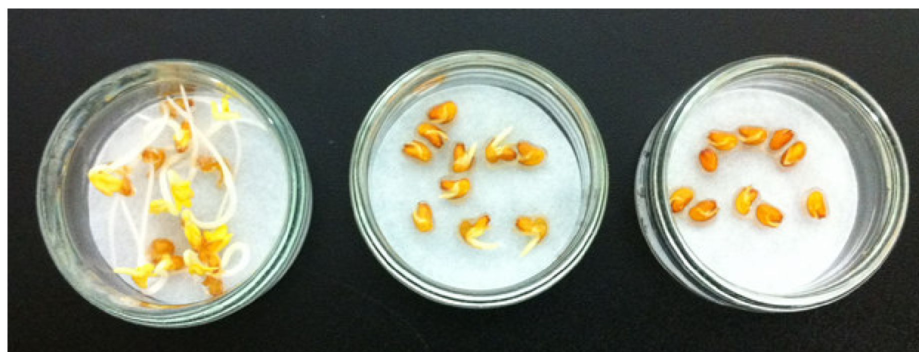

Figure 2. The seeds arrangement in Petri dishes (results of effects of β-sitosterol on cress at the concentration of 0, 0.1, and 1 µM from the left show, respectively, after 48 h incubation in dark at 25°C).

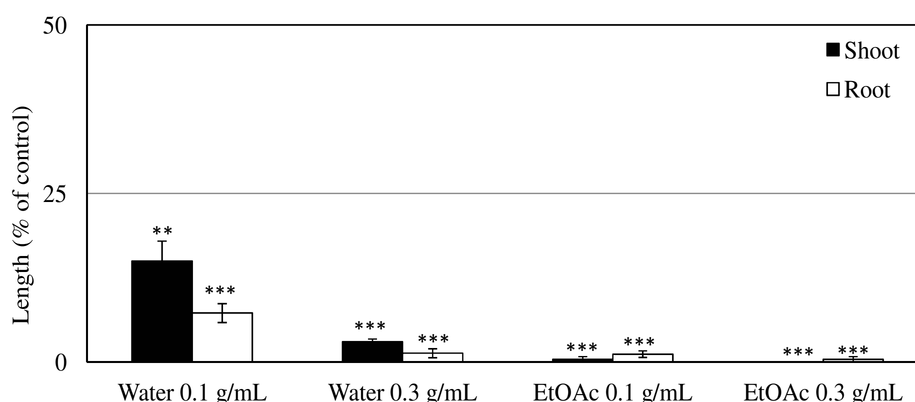

Figure 3. Effects of aqueous and ethyl acetate fractions obtained from *H. sabdariffa* on the shoot and root growth of cress seedlings. Means ± SE from three independent experiments with 10 seedlings for each determination are shown. Asterisk indicates significant difference between control and treatment: *$P<0.05$*, **$P<0.01$*, ***$P<0.001$* (Student's t-test).

n-hexane. The active residues were purified by columns of Sephadex LH-20, C_{18} Sep-Pak cartridges and the active substance was isolated by reverse phase HPLC at the retention time of 67.3 to 73.0 min, yielding an active compound (6.8 mg) as white residue.

Characterization of substance

The structure of the substance was identified by their spectroscopic data (^{1}H-, ^{13}C-NMR, HH-COSY and HMQC) and by comparison with published values. The ^{1}H NMR ($CDCl_3$) spectrum of the substance showed; δ 0.68 (3H, s, H-18), 0.81 (3H, d, J = 6.9 Hz, H-27), 0.83 (3H, d, J = 6.9 Hz, H-26), 0.84 (3H, t, J = 6.9 Hz, H-29), 0.92 (3H, d, J =6.2 Hz, H-21), 1.01 (3H, s, H-19), 3.52 (1H, quint, J =5.5 Hz, H-3), 5.35 (1H, m, H-6),

1.05-2.30 (30H, remaining protons). The ^{13}C NMR ($CDCl_3$) spectrum of the substance showed; δ 11.9 (C-18), 12.0 (C-29), 18.8 (C-21), 19.0 (C-27), 19.4 (C-19), 19.8 (C-26), 21.1 (C-11), 23.1 (C-28), 24.3 (C-15), 26.1 (C-23), 28.3 (C-16), 29.2 (C-25), 31.6 (C-2), 31.9 (C-7, 8), 34.0(C-22), 36.2 (C-20), 36.5 (C-10), 37.3 (C-1), 39.8(C-12), 42.3 (C-13), 45.9 (C-24), 50.2(C-9), 56.1 (C-17), 56.8 (C-14), 71.8 (C-3), 121.7 (C-6), 140.8 (C-5). From the comparison of data with the literature (22), these spectra indicated that this substance was β-sitosterol (Fig. 4).

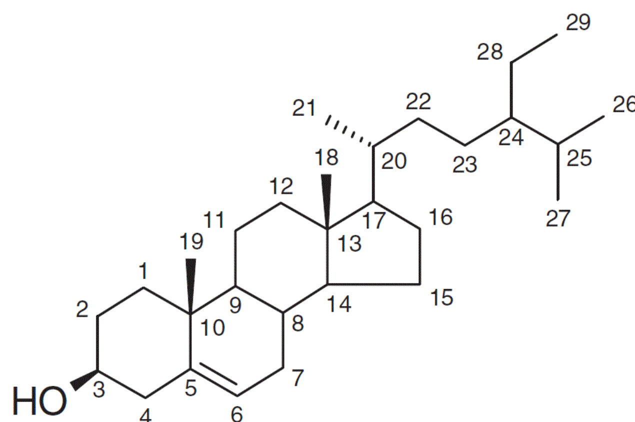

Figure 4. Chemical structure of β-sitosterol

Growth inhibitory activity of β-sitosterol

β-Sitosterol isolated from the *H. sabdariffa* inhibited the shoot and root growth of cress, lettuce, timothy, and Italian ryegrass at above 0.3 µM concentrations (Fig. 5). I_{50} values were calculated from the regression equation of concentration-response curves (Table 3). The I_{50} of roots and shoots of cress in the assay were 2.0 and 16.2 µM, respectively, and on lettuce were 406.7 and 52.4 µM, respectively. The I_{50} of roots and shoots of timothy were 10.2 and 11.7 µM, respectively and on those of Italian ryegrass were 52.4 and 61.0 µM, respectively. Comparing I_{50} values, effectiveness of β-sitosterol on shoots of timothy was much greater than on cress, Italian ryegrass, and lettuce and I_{50} values of β-sitosterol on roots of cress was much greater than on timothy, Italian ryegrass and lettuce.

Table 3. I_{50} values of β-sitosterol for shoots and roots of test plants

Test plant species	β-sitosterol I_{50} (µM)	
	Shoot	Root
Cress	16.2	2.0
Lettuce	52.4	406.7
Timothy	61.0	52.4
Italian ryegrass	11.7	10.2

I_{50} values were determined by a logistic regression analysis after bioassays ($P < 0.05$).

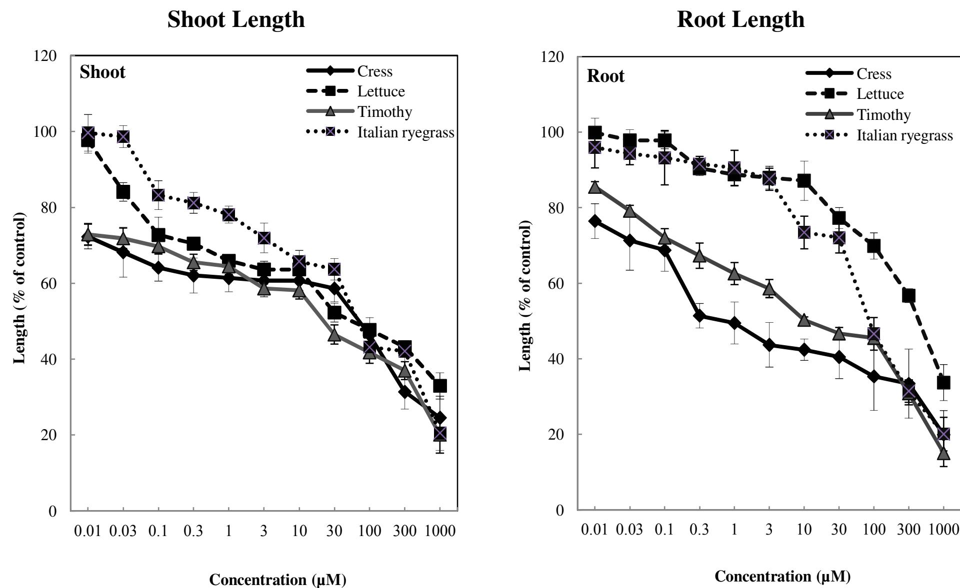

Figure 5. Effects of β-sitosterol on the shoot and root growth of cress, lettuce, timothy, and Italian ryegrass seedlings. Means ± SE from three independent experiments with 10 seedlings for each determination are shown.

DISCUSSION

The aqueous methanol extract was inhibitory to wide range of plant species, both monocotyledonous plants (timothy, crabgrass, Italian ryegrass, ryegrass, Chinese sprangletop, jungle rice, barnyardgrass and sand fescue) and the dicotyledonous plants (cress, lettuce, alfalfa and buckwheat). Such inhibition in growth of test plant species may be due to the presence of phytotoxins in *H. sabdariffa*. Similar results were reported by Randhawa *et al*. (30), who found that the germination of *Trianthema portulacastrum* was suppressed by higher concentration of sorghum water extract. The results are in agreement with earlier studies reporting that degree of inhibition increase with increased extract concentration (1,2,4,5,7,9,19,23,30).

The extracts of *H. sabdariffa* were more inhibitory to growth of roots than shoots of all test plant species. When compared to the control, the root length inhibition was greater than shoots. This may also be due to the contact of roots with the filter paper, leading to constant absorption of extract solution. Salam and Kato-Noguchi (33) reported that the extracts of allelopathic plants had more inhibitory effects on root growth than on hypocotyl growth, because root is the first organ to absorb allelochemical from the environment. Furthermore, the permeability of root tissues to allelochemicals was greater than shoot tissue (29). The inhibitory effects of *H. sabdariffa* were variable on test plant species. This unequal susceptibility to the extracts could be due to inherent differences in various biochemicals involved in the process. The aqueous methanol extract of *H. sabdariffa* plants showed allelopathic effects on all test plant species (Table 1). This study

has successfully isolated phytotoxins from *H. sabdariffa* plant using biological assay guided isolation approach. In addition, the inhibitory activity of β-sitosterol and its occurrence in *H. sabdariffa* suggest that β-sitosterol may play a role in the allelopathic properties of *H. sabdariffa*.

Triterpene β-sitosterol is a known plant sterol. It is a waxy substance white in colour. Only limited data are available concerning triterpene and steroid activity and plant growth development. Macías *et al*. (26) reported that lupanic triterpenes stimulates the germination of *Lactuca sativa* and *Hordeum vulgare*. Some steroids, such as chondrillasterol or amasterol affects the seed germination (6,11). The phytotoxicity of ergosterol peroxide isolated from rice (*Oryza sativa*) on barnyardgrass (*Echinochloa crus-galli*) was higher than commercial herbicide Logran (25). There are only a few reports about the allelopathic effects of β-sitosterol on crop plants (24). Fischer and Quijano (12) analyzed some bioactive compounds from the roots of *Ambrosia artemisiifolia* L. and found that β-sitosterol may act as allelochemicals via micelle formation with long-chain fatty acids. Kpoviessi *et al*. (21) isolated β-sitosterol from ethanol extract of *Justicia anselliana* and tested for the inhibitory effects on cowpea (*Vigna unguiculata*). β-sitosterol strongly inhibited the shoot growth of cowpea at 200×10^3 ug/mL .

The endogenous concentration of β-sitosterol was 32.7 µmol/kg or 13.6 mg/kg dry weight of *H. sabdariffa* in the present study. The threshold of *H. sabdariffa* for growth inhibition was 0.3 µM. Therefore, *H. sabdariffa* is considerably phytotoxic to the growth and development of test plant species. Further evaluation of allelopathic substance under field conditions and studies on the mode of action of this allelopathic substance are required for assist in the identification of novel target sites of action for weed control.

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