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Original article

BIOACTIVE ISOFLAVONES FROM *ERYTHRINA VARIEGATA* L.

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Abstract

Erythrina variegata L (Fabaceae) *has been investigated for isolation of secondary metabolites and evaluation of bioactivities. Previously, we reported re-isolation of alpinum isoflavone (**4**) and first isolation of 6-hydroxygenistein (**5**), 3β,28-dihydroxyolean-12-ene (**6**) and epilupeol (**7**) from a methanol extract of E. variegata. Now we report three further isoflavones, namely, scandenone (**1**), 4',5,7-trihydroxy-8-prenylisoflavone (**2**) and 4',5,7-trihydroxy-8-methylisoflavone (**3**), for the first time from this plant. Purified compounds (**2**, **4** and **5**) and crude fractions were subjected to free radical scavenging activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH). The crude methanol extract, n-hexane, carbon tetrachloride and chloroform soluble fractions showed moderate antioxidant activity (IC_{50}= 484.4-82.35 µg/ml), while the purified compounds, 4',5,7-trihydroxy-8-prenyl isoflavone alpinum isoflavone and 6-hydroxygenistein , exhibited high antioxidant activity having IC_{50} of 6.42, 8.30 and 8.78 µg/ml, respectively. At the same time the standard compound, tert-butyl-1-hydroxytoluene (BHT) demonstrated an IC_{50} of 5.88 µg/ml. In the β-glucosidase inhibitory bioassay, the crude methanol extract, n-hexane, carbon tetrachloride and chloroform soluble fractions of E. variegata revealed of 34.75, 95.04, 91.49 and 55.32%, inhibition respectively.*

Key words: *Erythrina variegata, Fabaceae, Alpinum isoflavone, Scandenone, 6-Hydroxygenistein, 4',5,7-Trihydroxy-methylisoflavone, 4',5,7-Trihydroxy-8-prenylisoflavone, β-Glucosidase inhibition, Antioxidant activity*

Erythrina variegata L.'dan Biyoaktif İzoflavonlar

*Erythrina variegata L. (Fabaceae) sekonder metabolitlerin izolasyonu ve biyolojik aktivitelerinin değerlendirilmesi için incelenmiştir. Daha önce E. variegata'nın metanol ekstresinden alpinum izoflavon'un (**4**) tekrar izole edildiği ve 6-hidroksigenistein (**5**), 3β,28-dihidroksiolean-12-en (**6**) ile epilupeol (**7**)'un ilk defa izole edildiği bildirilmiştir. Bu çalışmada izoflavon yapısında üç bileşik, skandenon (**1**), 4',5,7-trihidroksi-8-prenilizoflavon (**2**) ve 4',5,7-trihidroksi-8-metilizoflavon (**3**), bu bitkiden ilk olarak elde edilmektedir. Saf bileşikler (**2**, **4** ve **5**) ve ham fraksiyonlar 1,1-difenil-2-pikrilhidrazil (DPPH) serbest radikal yakalayıcı aktiviteleri (antioksidan aktivite) açısından incelenmiştir. Ham metanol ekstresi, n-hekzan, karbontetraklorür ve kloroformda çözünen fraksiyonlar orta derecede antioksidan aktivite (IC_{50}= 484.4-82.35 µg/ml) gösterirken, saf bileşikler, 4',5,7-trihidroksi-8-prenilizoflavon, alpinum izoflavon ve 6-hidroksigenistein sırasıyla 6.42, 8.30 ve 8.78 µg/ml IC_{50} değerleri ile yüksek antioksidan aktiviteye sahip oldukları bulunmuştur. Standart bileşik olan t-butil-1-hidroksitoluen (BHT)'in IC_{50} değeri ise 5.88 µg/ml olarak bulunmuştur. β-glukozidaz inhibitör biyolojik analizinde, E. variegata'nın ham metanol ekstresi, n-hekzan, karbon tetraklorür ve kloroformda çözünen fraksiyonları sırasıyla % 34.75, 95.04, 91.49 ve 55.32 inhibisyon göstermişlerdir.*

Anahtar kelimeler: *Erythrina variegata, Fabaceae, Alpinum izoflavon, Skandenon, 6-Hidroksigenistein, 4',5,7-Trihidroksimetilizoflavon, 4',5,7-Trihidroksi-8-prenilizoflavon, β-Glukozidaz inhibisyonu, Antioksidan aktivite*

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INTRODUCTION

Natural products have been the single most productive source of leads for the development of drugs. Various screening approaches like β-glucosidase inhibition, antioxidant and many new bioassays are being developed to improve the ease with which natural products can be used in drug discovery campaigns (1). The enzyme β-glucosidase is known to be involved in glycoprotein processing. So the β-glucosidase inhibitors are potentially useful as antiviral, antiadhesive, antibacterial, antimetastatic or immunostimulatory agents (2-4). On the other hand, oxidative stress has been known to contribute to the general decline in cellular functions that are associated with many human diseases, including Alzheimer's disease, amyotrophic lateral sclerosis, parkinson's disease, atherosclerosis, ischemia, degenerative changes of the human temporomandibular-joint, cataract formation, macular degeneration, retinal damage, rheumatoid arthritis, multiple sclerosis, muscular dystrophy, human cancers as well as the aging process itself. In this case, antioxidants play a vital role to combat these diseases (5). Recently, drugs from natural sources are becoming popular due to its availability, cost effectiveness, exploration of new chemical entities, counteraction of drug resistance etc.(1,6) Keeping these in mind, as a part of our continuous studies of Bangladeshi medicinal plants, we investigated *Erythrina variegata* L.

Erythrina variegata L. (Fabaceae) known as Mandar in Bengali is a medium-sized deciduous small tree with prickly stems and branches, leaves with triangular leaflets and large coral red flowers and grows all over Bangladesh. Medicinally the bark of the plant is astringent, febrifuge, anti-bilious and anthelmentic. It is also useful in opthalmia and skin diseases. The leaves are used in fever, inflammation and joint pain. The juice of the leaves is used in earache, toothache (7), constipation, cough (8) and also known to stimulate lactation and menstruation. To date, a variety of secondary metabolites including orientanol B, erycristagallin, cristacarpin, sigmoidin K, 2-(γ,γ-dimethylallyl)- 6a-hydroxyphaseollidin, erystagallin A (9), eryvarins A and B (10), bidwillon B (11), eryvarinols A and B (12), eryvarins F and G (13), alpinum isoflavone, isococculinine, decarbomethoxyerymelanthine, erysodienone, erythritol, erysodine (14), erysovine, stachydrine, sterols, fixed oils and fatty acids (15) have been reported from *E. variegata*. Previously, we reported re-isolation of alpinum isoflavone (**4**) and first isolation of 6-hydroxygenistein (**5**), 3β,28-dihydroxyolean-12-ene (**6**) and epilupeol (**7**) from a methanol extract of *E. variegata* (7). As a continuation of our work with this plant, we now report three further isoflavones, namely scandenone (**1**), 4′,5,7-trihydroxy-8-prenylisoflavones (**2**) and 4′,5,7-trihydroxy-8-methyl isoflavone (**3**), for the first time from this species.

The aim of the present investigation was to isolate the secondary metabolites as well as to evaluate of the antioxidant and β-glucosidase inhibitory activities of extracts of *E. variegata.*

MATERIALS AND METHODS

General experimental procedures

^{1}H NMR spectra were recorded on a Bruker AMX-400 (400 MHz) instrument, using $CDCl_3$ and the spectra were referenced to the residual nondeuterated solvent signal. Column chromatography (CC) was carried on Merck Si gel 60 H. TLC and PTLC were conducted on glass plate coated with Merk Si gel 60 PF_{254} at a thickness of 0.5 mm. Spots on TLC and PTLC plates were visualized under UV light (254 and 366 nm) and by spraying with 1% vanillin in sulphuric acid, followed by heating.

Stem bark of *E. variegata* was collected from Dhaka in September 2004. Plant material was identified by Mr. Sarder Nasir Uddin, Scientific officer, Bangladesh National Herbarium. Voucher specimen (DACB-32884) are kept at the Bangladesh National Herbarium, Bangladesh.

Extraction and Isolation

The powdered stem bark (750 gm) of *E. variegata* was soaked in 1.5 L of methanol for 7 days and then filtered through a cotton plug followed by Whatman filter paper number 1. The extract was concentrated at reduced pressure with a vacuum rotary evaporator at 40°C. A portion (5 gm) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning method (16) to afford *n*-hexane (0.55 gm), carbon tetrachloride extract (0.67 gm), chloroform (1.50 gm) and aqueous soluble (2.00 gm) materials. The chloroform and carbon tetrachloride soluble partitionates were separately chromatographed over silica gel (Kiesel gel 60H, mesh 70-230) and the columns were eluted with *n*-hexane followed by mixtures of *n*-hexane and ethyl acetate in order of increasing polarities. Preparative TLC (toluene:EtOAc, 85:15) of fractions obtained from chloroform soluble extractive eluted with 20% ethyl acetate in *n*-hexane provided compound **1** (1.0 mg) in pure form. Similar column chromatographic separation of the carbon tetrachloride soluble materials eluted with 35-45% ethyl acetate in *n*-hexane yielded compounds **2** (2.0 mg) and **3** (2.0 mg). Evaporation of solvents from column fractions eluted with 25% ethyl acetate in *n*-hexane gave compound **4** (4 mg) as yellow solid. On the other hand, preparative TLC (toluene-EtOAc, 80:20) of the fractions eluted with 50% EtOAc in *n*-hexane provided compound **5** (1.0 mg).

Scandenone (**1**)

^{1}H NMR (400 MHz, $CDCl_3$): δ 13.17 (1H, s, OH-5), 7.88 (1H. s, H-2), 7.39 (2H, d, *J*= 8.0 Hz, H-2′,H-6′), 6.90 (2H, d, *J*=8.0 Hz, H-3′, H-5′), 6.72 (lH, d, *J*= 10 Hz, H-4″), 5.60 (lH, d, *J*=10 Hz, H-3″), 5.17 (1H, t, *J*=7.2 Hz, H-2‴), 4.32 (lH, s, OH-4′), 3.39 (2H, d, *J*=7.2 Hz, H_2-1‴), 1.80, 1.68 (6H, 2 x s, Me_2-3‴), 1.46 (6H, Me_2-2″).

4′,5,7-Trihydroxy-8-prenylisoflavone (**2**)

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.71 (1H, s, H-2), 7.24 (2H, d, *J*= 8.0 Hz, H-2′, H-6′), 6.79 (2H, d, *J*= 8.0, H-3′, H-5′), 6.28 (1H, s, H-6′), 5.17 (1H, t, *J*=6.5 Hz, H-2″), 3.28 (2H, d, *J*=6.5, H-3″), 1.70 (3H, s, H-5″), 1.59 (3H, s, H-4″).

4′,5,7-Trihydroxy-8-methylisoflavone (**3**)

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.83 (1H, s, H-2), 7.40 (2H, d, *J*= 8.5 Hz, H-2′, H-6′), 6.98 (2H, d, *J*=8.5 Hz, H-3′, H-5′), 6.36 (1H, s, H-6), 2.61 (3H, s, H-8).

Antioxidant activity

The antioxidant activity of the extracts and purified compounds **2**, **4** and **5** on the stable radical DPPH was determined by the method of Brand-Williams et al. (1995) (17). For crude extracts, 2.0 mg of each of the extracts was dissolved in methanol. Solution of varying concentrations such as 500 μg/ml, 250 μg/ml, 125 μg/ml, 62.50 μg/ml, 31.25 μg/ml, 15.62 μg/ml, 7.8125 μg/ml, 3.91 μg/ml, 1.95 μg/ml and 0.98 μg/ml were obtained by serial dilution technique. The stock solution of the pure compounds were prepared in methanol from which dilutions were carried out to obtain concentrations of 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 μg/ml. The samples were mixed with a DPPH-methanol solution (20 mg/L) in the ratio of 1: 1.5 and were allowed to react for 20 minutes in absence of light. Absorbance values were determined at 517 nm using UV-Vis spectrophotometer and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

$$\% \text{ inhibition} = [1- (ABS_{sample} / ABS_{control})] \times 100$$

Then % inhibitions were plotted against respective concentrations used and from the graph obtained, the IC_{50} was calculated. *Tert*-butyl-1-hydroxytoluene (BHT), a potential antioxidant, was used as positive control.

β-glucosidase inhibitory activity

β-glucosidase inhibitory activity of the crude extracts was measured sepctrophotometrically using *p*-nitrophenyl-*β*-D-glucopyranoside (18,19). A 0.4 ml of substrate (*p*-nitrophenyl-*β*-D-glucopyranoside, 2 mg/ml), 1 mg of extract and 0.4 ml of pH 5 phosphate buffer (0.1M potassium hydrogen phthalate - NaOH) were placed in a test tube and incubated at 37 °C for 10 min. Then, 0.2 ml of enzyme (*β*-glucosidase, 20 mg/ml) solution was added and the mixture was incubated for another 30 minutes at 37 °C. After this, the reaction was terminated by adding 2.6 ml of pH 10 buffer (2M glycine–NaOH). In the positive control, a mixture of solvents was added instead of the extract, while in the negative control, pH 10 buffer was added at the beginning of the test in order to block enzyme activity. Absorbance values of the test samples were measured at 410 nm and the activity was calculated with the help of the following formula:

$$\% \text{ Enzymatic inhibition} = 100 - [(ABS_{sample} - ABS_{negative\ control}) / ABS_{positive\ control}] \times 100$$

Statistical analysis

The tests were conducted in triplicate for each of the bioassays. The IC_{50} and % enzymatic inhibition were calculated as mean ± SD (n=3) for the antioxidant screening and β-glucosidase inhibitory bioassay, respectively.

RESULTS AND DISCUSSION

Repeated chromatographic separation and purification of the chloroform and carbon-tetracholoride soluble fractions of a methanolic extract of the stem bark of *E. variegata* yielded three compounds. The structures of the isolated compounds **(1-5)** (Figure 1) were identified by ^{1}H NMR data.

The ^{1}H NMR spectrum of compound **1**, (400 MHz, $CDCl_3$) revealed well resolved signals between δ 1.20-7.90 and at δ 13.17 typical of an isoflavone type carbon skeleton having a pyran ring. Thus, the ^{1}H NMR spectrum showed a pair of doublets (*J*=10 Hz) centered at δ 5.60 and 6.72 and a sharp singlet of six proton intensity at δ 1.46, which were assigned to a 2,2-dimethylchromene ring system. The characteristic C-2 proton of the isoflavone skeleton was evident as a singlet at δ 7.88 (1H). The ^{1}H NMR spectrum also displayed a pair of doublets (*J*= 8.0 Hz), each integrating for two protons, at δ 6.90 and 7.39, which were assigned to the H-3′ & H-5′ and H-2′ & H-6′ of the *para*-disubstituted aromatic nucleus. The relatively upfield resonance (δ 6.90) of H-3′ and H-5′ suggested the presence of an oxygenated substituent at C-4′, probably as a hydroxyl group. This was substantiated by the presence of a broad singlet at δ 4.32 (1H), assignable to the hydroxyl group proton. In addition to this, the ^{1}H NMR spectrum of compound **1** displayed two broad singlets of three proton intensity each at δ 1.68 and 1.80, a triplets (*J*= 7.0 Hz) of one proton intensity at δ 5.17 and a two protons doublet (*J*= 7.0 Hz) at δ 3.39. These suggested the presence of a 3,3-dimethylallyl side chain, which could be attached to C-8 of the isoflavone nucleus. The remaining signal at δ 13.17 (1H) could be attributed to the chelated hydroxyl group proton at C-5. On this basis and by comparison of these values with published data for scandenone (20), the identity of compound **1** was confirmed as scandenone **(1)**. Although it has previously been reported from plants of the genera *Milletia, Derris, Maclura* etc. (14), this is the first report of its isolation from *E. variegata.*

The ^{1}H NMR spectrum of compound **2** (400 MHz, $CDCl_3$ + few drops of CH_3OD) showed well resolved proton resonances between 1.50 and 8.00 ppm which were similar to that of compound **1**, except the resonances assignable to the pyran ring protons. It showed a one proton singlet at δ 7.71 typical of H-2 of the isoflavone skeleton. The doublets (J= 8.0 Hz) centered at δ 6.79 and 7.24, each of two proton intensity, could be assigned to a *para* di-substituted aromatic ring protons at C-3′ & C-5′ and C-2′ & C-6′, respectively. The relatively upfield shift of C-3′ & C-5′ protons demonstrated an oxygenated functionality at C-4′. The lone aromatic proton at δ 6.28 can be attributed to H-6. Furthermore, the ^{1}H NMR spectrum displayed two broad singlets at δ 1.59 and 1.70, a two proton doublet (J= 6.5 Hz) at δ 3.28 and a triplet (J= 6.5 Hz) integrating for one proton at δ 5.17. These signals are suggestive of the presence of a 3,3-dimethylallyl side chain at C-8 of the isoflavone skeleton. The above spectral data are consistent with 4′,5,7-trihydroxy-8-prenylflavone (**2**) (14). Although, the compound has been isolated from many pants (14), this is the first report of its occurrence from *E. variegata.*

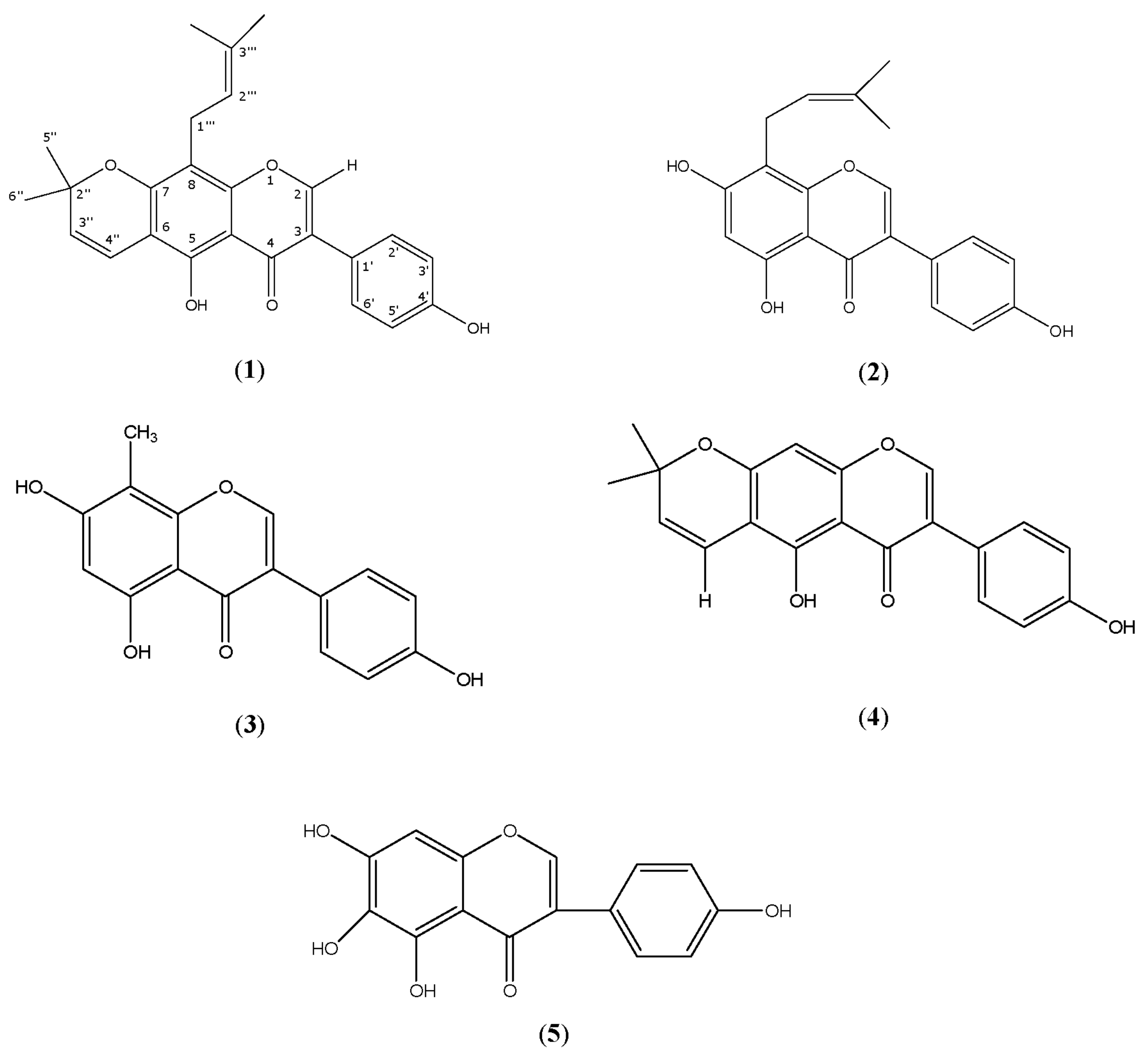

Figure 1. Investigated compounds.

The ^{1}H NMR spectrum of compound **3** was almost identical to that of compound **2** (4′,5,7-trihydroxy-8-prenylflavone). However, the signals assignable to the prenyl side chain in **2** were

absent and were replaced by a three proton resonance at δ 2.61. This demonstrated the presence of a methyl group at C-8 in compound **3** instead of a prenylated side chain in compound **2**. The ^{1}H NMR spectrum of compound **3** (400 MHz, $CDCl_3$) also showed a one proton singlet at δ 7.83 typical of H-2 of the isoflavone type carbon skeleton. The doublets (*J*= 8.5 Hz) centred at δ 6.98 and δ 7.40, each of two proton intensity, could be assigned to a *para* di-substituted aromatic ring protons at C-3′ & C-5′ and C-2′ & C-6′, respectively. An aromatic proton at δ 6.36 can be attributed to H-6. The above spectral data are consistent with 4′,5,7-trihydroxy-8-methylisoflavone (**3**) (14).

In the antioxidant study, the free radicals generated by DPPH were scavenged by the plant extracts to various degrees. Among the crude samples, the chloroform soluble partitionate of the stem bark showed the highest free radical scavenging activity, with IC_{50} value 82.35 µg/ml. At the same time, the methanolic extract exhibited an IC_{50} value of 85.03 µg/ml. On the other hand, the carbon tetrachloride and *n*-hexane soluble fractions of methanolic extract showed 50% inhibition at a concentration of 163.9 and 484.4 µg/ml respectively. The purified compounds showed high antioxidant activity with IC_{50} values close to IC_{50} of BHT. The IC_{50} values of BHT, compound **2**, **4** and **5** were found to be 5.88, 8.30, 8.78 and 6.42 µg/ml, respectively (Table 1).

Table 1. Antioxidant activity of the extracts of *E. variegata.*

Samples	**IC_{50} (µg/ml)***
Methanol extract of stem bark	85.03 ± 1.20
n-Hexane soluble fraction of methanolic extract	484.4 ± 1.60
CCl_4 soluble fraction of methanolic extract	163.9 ± 1.33
$CHCl_3$ soluble fraction of methanolic extract	82.35 ± 1.73
4′, 5, 7-Trihydroxy-8-prenylisoflavone (**2**)	6.42 ± 1.36
Alpinum isoflavone (**4**)	8.30 ± 1.41
6-Hydroxygenistein (**5**)	8.78 ± 1.49
Tert-butyl-1-hydroxytoluene	5.88 ± 1.11

*The values are expressed as mean ± S.D. (n=3)

In addition, extracts capable of inhibiting β-glucosidase activity can be used against various diseases related with glycoprotein processing. It has been reported that an inhibitory activity of 50% can be considered significant for plant extracts (2). In this investigation, the test samples showed significant β-glucosidase enzyme inhibition. Among them, the carbon tetrachloride and *n*-hexane soluble fractions of methanolic extract were found to be potent showing 91.49% and 95.04% β-glucosidase inhibition, respectively (Figure 2). The crude methanolic extract showed poor inhibitory activity, while the chloroform soluble fraction showed moderate inhibition of β-glucosidase.

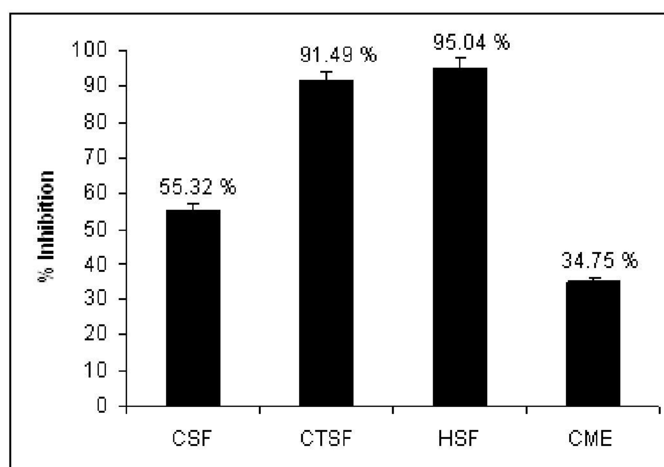

Figure 2. Percentage inhibition of β-glucosidase by extracts of *E. variegate*. Each bar indicates the mean ± S.D. (n=3); CSF: chloroform soluble fraction of the methanol extract; CTSF: Carbon tetrachloride fraction of the methanol extract; HSF: *n*-Hexane soluble fraction of the methanol extract; CME: crude methanol extract.

It is evident from the bioassays, that the purified isoflavonoids (**2**, **4** and **5**) as well as crude methanol extract and chloroform soluble partitionate of *E. variegata* demonstrated significant antioxidant activities. At the same time, *n*-hexane and carbon tetrachloride soluble partitionates of the methanol extract demonstrated very strong β-glucosidase enzyme inhibitory activity. These bioactivities support the folk uses of *E. variegata* in various diseases like skin diseases, joint pain etc. (2-5).

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