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An Antioxidant Organic Compound Isolated from the Stem of *Hypericum calycinum* L. (Pyin-nyar-lin-kar)

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Abstract

In the present study, stem of *Hypericum calycinum* L. (Pyin-nyar-lin-kar in Myanmar), which is mainly used in the treatment of malaria in Myanmar Traditional Medicinal System, was selected for chemical investigation. The preliminary phytochemical screening of the stem of this plant was performed. Moreover, ant bacterial tests of the crude extracts of this plant sample were done by agar well diffusion method in various solvents on six selected organisms. The antioxidant activities of three crude extracts of *Hypericum calycinum* L. were determined by using 1-1-diphenyl-2-picryl hydrazyl (DPPH) assay. In addition, the pure colourless crystal was isolated from the ethyl acetate extract of the stem of *Hypericum calycinum* L. by using Thin Layer and Column Chromatographic methods. The phytochemical test of this compound gave positive result for flavonoid group only. The molecular formula of this compound could be assigned as $C_{25}H_{28}O_6$ based upon some advanced spectroscopic methods. The complete structure of this new compound was elucidated on the basis of 1D and 2D-NMR analysis as well as mass spectrometry (EI and HR-MS). The scientific name of this elucidated compound is named as 5-hydroxy-2*S*-[2,4,6-trihydroxy)-3-(2-isoprophenyl-5-methyl-hex-4-enyl) phenyl]-chroman-4 one. In order to know the antioxidant activity of this compound, Oxygen Radical Absorbance Capacity (ORAC) assay was performed. Moreover, cytotoxic activity of this compound was done by Colorimetric MTT (tetrazolium) assay on HeLa human cervix carcinoma cell and Coca-2 human epithelial colorectal adenocarcinoma cell.

Key words: *Hypericum calycinum* L., Pyin-nyar-lin-kar, flavanone, DPPH, ORAC assay, MTT assay.

Introduction

It is known that many infectious diseases can be treated with plant materials throughout the history of mankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries. Plants still continue to be almost the exclusive source of drugs for the majority of the world's population.

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Myanmar has a long history of health care system by herbal and medicinal plants as a national heritage. In Myanmar, the study of indigenous medicinal plants and their usage in therapy plays a very important role. These plants may have biologically active principles. The plant kingdom constituents are invaluable source of new chemical products which may be important due to their bioactive properties and their potential uses in medicine.

In recent years there is a considerable interest and increase in the use of natural antioxidants. Antioxidants are any substance which inhibits a free radical reaction, that is a cause of numerous diseases. Free radicals can cause lipid per-oxidation in foods, which leads to their deterioration. In addition, reactive oxygen species have been implicated in more than 100 diseases including malaria, acquired immunodeficiency syndrome, heart diseases, stroke, arteriosclerosis, diabetes and cancers. Therefore, research for the determination of the natural antioxidants source is important. Cytotoxic drug is one that damages or destroys cells and is used to treat various cancers, either with or without the use of radiotherapy. Cytotoxic drugs offer successful treatment in some conditions and help reduce symptoms and prolong life in others. They destroy cancer cells by inhibiting cell division i.e., they are antimitotic (Kiendrebeogo, *et al.*, 2005).

The leaves of *Hypericum calycinum* L. containing xanthones have been reported to possess anti-malaria properties (Konovalova, 2007, Decosterd, *et al*, 1991 and 1988). However, the flavanone compound has not been previously isolated from the stem of *Hypericum calycinum* L.

Therefore, in the present study, the antioxidant activities of some crude extracts of stem of *Hypericum calycinum* L were determined and the most antioxidant extract was used for further isolation and purification of organic compound using advanced chromatographic methods. Moreover, pure constituent was detected for its main antioxidant flavanone and for its cytotoxicity.

Materials and Methods

General

Melting point is uncorrected and was measured in open capillary tubes, using a Gallenkamp melting point apparatus. ^{1}H NMR spectral data

were obtained from a Brucker Advance 600 Spectrometer (600 MHz) and ^{13}C NMR was recorded by Brucker Advance 600 Spectrometer (150 MHz). TLC was performed on silica gel F254 plates (Merck). For detection, iodine vapour or UV-Lamp (lambda-40) Perkin-Elmer Co. England was used. Silica gel chromatography utilized silica gel 60 (70-230 mesh). FT-IR data was collected by FT-IR Spectrometer (Shimadsu, Japan). EI-MS and HR-MS spectral data were recorded using Mass Spectrometers (Finnigan MAT SSQ 710, USA and Finnigan MAT 95, USA). UV measurement was performed on a Jasco V-570 UV/VIS Spectrophometer. For MTT and ORAC assay, Spectrafluor Plus plate reader (Tecan, Germany) was used.

Sampling

The stems of *Hypericum calycium* L. were collected from Kyauk Me Township, Shan State. It was identified by the botanists of Department of Botany, University of Mandalay. The stems were cut into small pieces and air-dried at the room temperature and then stored in an air-tight container.

Preliminary Phytochemical Screening was done on this sample according to the standard methods (Priestman, 1953, Finar, 1964, and Vogel, 1956, 1966).

Antimicrobial Activities on Crude Extract

Antibacterial tests were done for the determination of antibacterial activities of plant extract *Hypericum calycinum* L. (Pyin-nyar-lin-kar). Agar well diffusion method was used and tested on six types of organisms (*Bacillus subtilis, Bacillus aureus Pseudomonous aeruginosa, Bacillus pulmalis Candida albican, Mycobacterium species*).

Preliminary Screening of Radical Scavenging of Crude Extracts for Stem of *Hypericum calycinum* L. by DPPH Assay was measured as described by Velaskez et al. (2003). The sample solution was prepared by mixing test solution with three different extracts, ethanol, ethyl acetate and pet ether (1.5 cm^3) with 60 μM DPPH solution. After shaking, the mixture was incubated for 15 min in the darkness at the room temperature and then absorbance was measured at 517 nm. The difference in absorbance between a test sample and a control (ethanol) was taken as the activity. The activity was expressed as IC_{50} value (μg/mL: 50 % inhibitory concentration). Ascorbic acid (IC_{50} value 1.88 μg/mL) served as standard. Each assay was conducted 3 times.

Percolation and Extraction

The air-dried sample was percolated with 95% ethanol for two months and then filtered off and filtrate was evaporated. EtOH extract was separated by ethyl acetate solvent. It was evaporated for further isolation.

Isolation

The ethyl acetate extract (2.4g) was fractionated by column chromatogram over silica-gel (70-230 mesh) eluting with various ratio of n-Hexane and ethyl acetate from non-polar to polar. Totally 190 fractions were obtained and each fraction was checked by TLC. The fractions with same R_f value were combined and it gave nine combined fractions. Among these fractions, two fractions (fraction No. IV and VII) were major portions because they give characteristic spots on each TLC plates and the yields are high. These two portions were fractionated again by using micro column. From the fraction No. IV, the pure colourless crystal (**TW-1**) (20.1 mg, yield 56% based upon ethyl acetate crude extract) was obtained.

The antioxidant activity of isolated pure compound (**TW-1**) was determined by oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay (Decker, *et al.*, 2008). The ORAC assay measures antioxidant scavenging activity against peroxyl redical induced by 1,1'-azobis (2-amidinopropane) dihydrochloride (AAPH) at 37 °C. AAPH (0.414 g) was dissolved in 10 ml of 75 mM phosphate buffer (p^H 7.4) to a final concentration of 153 mM and made fresh daily. A fluorocein stock solution (4×10^{-3} mM) was made in 75 mM phosphate buffer (p^H 7.4) and stored wrapped in foil at 5°C. Immediately prior to use, the stock solution was diluted 1:1000 with 75 mM phosphate buffer (p^H 7.4). The diluted sodium fluoroscene solution was made fresh daily. In regards to the plate usage, the exterior wells were not used for experimental determination. In order to avoid the temperature effect, the interior wells are used for experimental purpose. These exterior wells were filled with 300 µL of distilled water. To all experimental wells, 150 µL of sodium fluoresein solution was added. In addition, blank wells received 25 µL of 75 mM phosphate buffer (p^H 7.4), while the standards received 2.5 ml of Trolox™ dilution and samples received 25 µL of sample. The plate was allowed to equalibrate by incubating for a minimum of 30 minutes in the plate reader. Reactions were initiated by the addition of 25 µL AAPH solution using the microplate reader's injector for a final volume of 200 µL. The fluoresein was then monitored kinetically with data taken every minute.

The AUC (Area Under Curve) and the Net AUC of the standards and sample were determined as:

$$AUC = 1 + \sum_{i=0}^{i=i} \frac{f_i}{f_0} \quad (1)$$

where f_o is the initial fluorescence at 0 min and f_i is the fluorescence at time i.

The net AUC for a sample was calculated as follows:

$$\text{Net AUC} = AUC_{sample} - AUC_{blank} \quad (2)$$

The standard curve was obtained by plotting the Net AUC of different Trolox® standard curve. Final result was in μM of Trolox equivalent/ μM of pure compound.

In Vitro Cytotoxicity Test of the Pure Compound

Cytotoxicity effect of this compound against HeLa human cervix carcinoma cell and Caco-2 human epithelial colorectal adeno carcinoma cell were evaluated using colorimeteric methylthiazoletetra zolium (MTT) assay (Mosmann, 1983). In brief, cells were seeded in 96 well plates at the density of 5×10^3 per well and cuttured for 24 hours. Subsequently cells were incubated for another 24 hours either with medium only or medium supplemented with solvent only. After treatment, 10 μL MTT (Sigma, Schnelldrof, Germany) solution (5 mg/mL PBS) was added to each well and cells were incubated for another 3 hours at 37°C. Subsequently 100 μL lysis buffer (10% SDS, pH 4.1) was added and formazen allowed to dissolve overnight. Absorbance at 550 nm was determined with a multi well plate reader.

Structure Elucidation

Spectroscopic data of isolated compound (**TW-1**) were recorded by using advanced spectroscopic methods such as ^{1}H NMR, ^{13}C NMR, DEPT, UV-VIS, FT-IR, HSQC, DQF-COSY, HMBC, EI-MS, and HR-MS.

CD (Circular dichroism) spectrum was measured to know the absolute configuration of chiral center of pure compound.

Results and Discussion

Preliminary phytochemical analysis was performed in order to know the different types of chemical constituents present in the stem of *Hypericum calycium* L. According to this result, flavonoid, terpene, phenol, and alkaloid were present in this plant. From the screening of microbial activities of selected plant, aqueous ethanol, ethyl acetate, and acetone plant extracts showed high activities against all tested organisms. The antioxidant activities of crude extracts (*in vivo)* were done by using 1, 1-diphenyl-2-pecrylhydrazyl (DPPH) assay. The ascorbic acid was used as a standard compound in this assay. The purple color rapidly faded when DPPH encountered any radical scavengers (Velaskez, *et al.*, 2003). Crude aqueous pet ether extract shows moderate DPPH radical scavenging activity with an IC_{50} value of 5.71μg/mL. In comparison with the ethanol crude extract which exhibits an IC_{50} value of 3.67 g/mL, the ethyl acetate extract has the highest antioxidant activity (IC_{50} =2.04 μg/mL). Bio-guided fractionation led to the main radical scavenging of the stem of *Hypericum calycium* L. in the ethyl acetate fraction. The pure flavanone typed compound (**TW-1**) was isolated from the most antioxidant ethyl acetate fraction of the stem of *Hypericum calycinum* L. using Thin Layer and Column Chromatographic techniques. The yield of this pure compound (**TW-1**) was found to be 20.1 mg, 0.56% based upon the ethyl acetate extract. The melting point of **TW-1** is (174-176 °C). According to the phytochemical test, it showed positive reaction for flavonoid.

FT-IR spectrum of **TW-1** represents OH stretching vibration band at 3371.3 cm^{-1}. The band which appears at 2923.3 cm^{-1} is due to symmetrical and stretching vibration of saturated hydrocarbons. Typical flavonoid C=O stretching vibration band is observed at 1635.5 cm^{-1}. The band at 1604.7 cm^{-1} is due to aromatic C=C stretching vibration of alkenic group. This **TW-1** compound in methanol shows a UV maximum at 292 nm and inflection at 334 nm. These UV absorptions have been assigned Л to Л* and n to Л* origins, respectively. The ^{1}H NMR Spectrum (600 MHz) of compound **TW-1** shows total number of protons. According to these data, this compound contains 24 protons. From ^{13}C NMR (150 MHz), there are total numbers of 25 carbons. Table 1 shows the data of DEPT (Distortionless Enhancement by Polarization Transfer) which represents the kinds of carbons and protons.

Table 1. Assignment of DEPT spectral data

No.	Assignment	No. of carbons	No. of protons
1	3- sp^3 methyl carbons	3	9
2	3- sp^3 methylene carbons	3	6
3	2- sp^3 methine carbons	2	2
4	1- sp^2 methylene carbon	1	2
5	5- sp^2 methine carbon	5	5
6	11-sp^2quaternary carbons	11	-
	Total	25	24

Splitting patterns (t at δ 6.98 ppm, d at δ 6.35 ppm) and coupling constant J (8.2 Hz) of proton NMR spectrum (**TW-1**) show the tri-substituted aromatic fragment including ring A as shown in Fig. 1. According to the ^{1}H NMR, HSQC, and HMBC spectra, another ring B including singlet aromatic methine proton (δ 5.89 ppm) was assigned. Moreover, unusual isoprene back bone skeleton was found being attached to the ring B utilizing DQF-COSY, HMBC, HSQC, and DEPT spectral data.

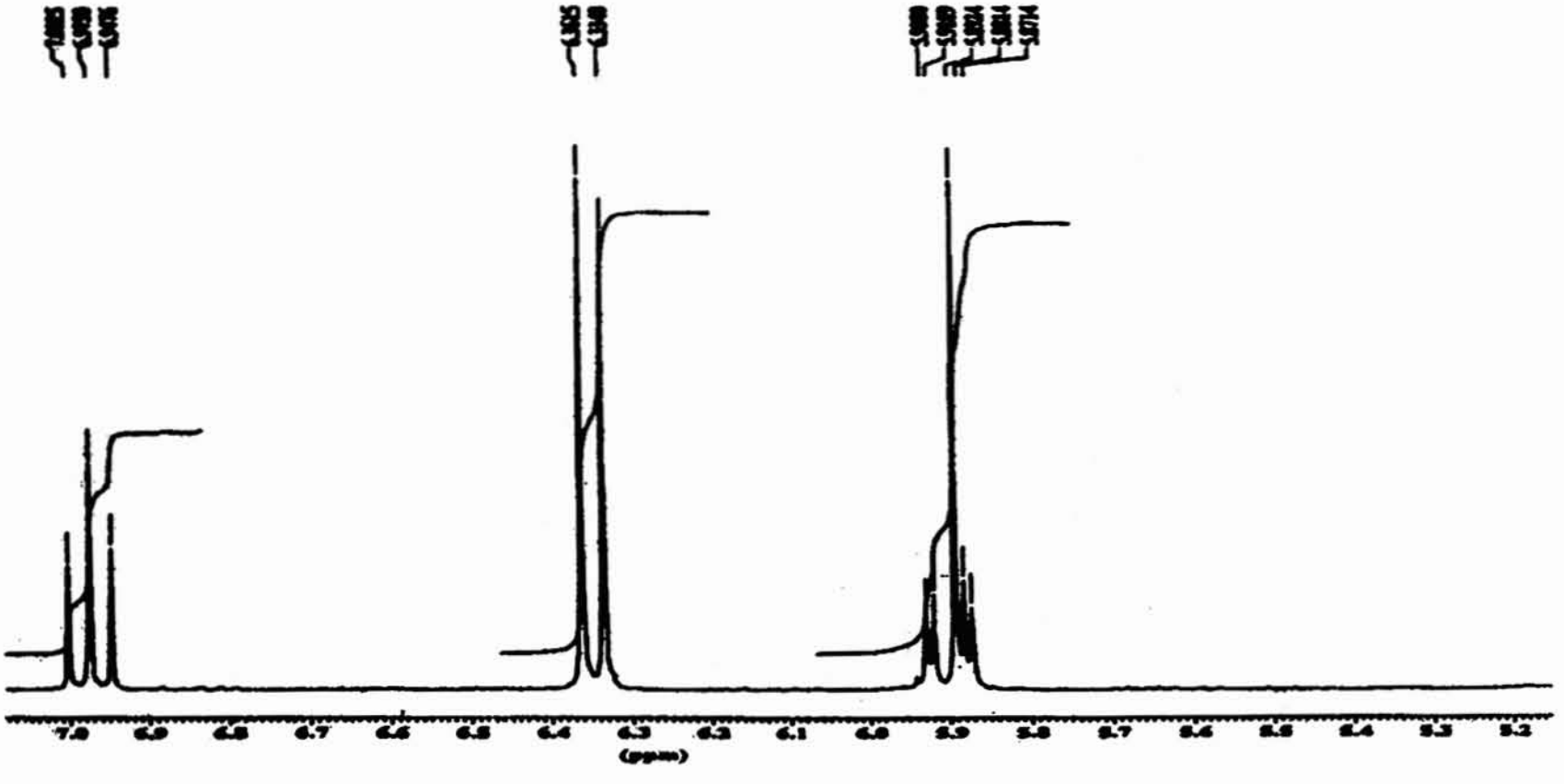

Fig. 1. ^{1}H NMR spectrum of compound **TW-1** in MeOD

EI-MS spectrum indicates that real molecular mass of **TW-1** is 424. On the basis of FT-IR, 1D NMR, 2D NMRs and EI-MS, the following new compound (Fig. 2) which is flavanone typed compound containing two chiral centers was completely elucidated. The existence of isoperene group was confirmed by EI-MS fragmentation which showed the peak at m/z 301. The deduced molecular formula $C_{25}H_{28}O_6$ is consistent with the HR-MS spectral data (m/z 424.1882, calcd 424.1879). CD spectrum was then applied to this isolated flavanone compound in order to prove its absolute configuration at C-2 chiral center. This flavanone showed a split CD curve having a negative Cotton effect at 292 nm and a positive one at 334 nm indicating the absolute configuration at the C-2 position to be *S*. The large coupling constants $J_{2,3}$ with the value of 14.39 *Hz* indicates that natural flavanone are favored in conformation with the C-2 aryl group equatorial (Fig. 2). However, another chiral center at isoprene skeleton could not be deduced due to the free rotation of single bond. Scifinder program provides that this elucidated flavanone containing novel isoprene skeleton in ring B is new compound. This new flavanone compound was isolated for the first time from *Hypericum calycium* L. The scientific name of this elucidated compound is 5-hydroxy-2*S*-[2,4,6-tri hydroxy)-3-(2-isoprophenyl-5-methyl-hex-4-enyl) phenyl]-chroman-4 one.

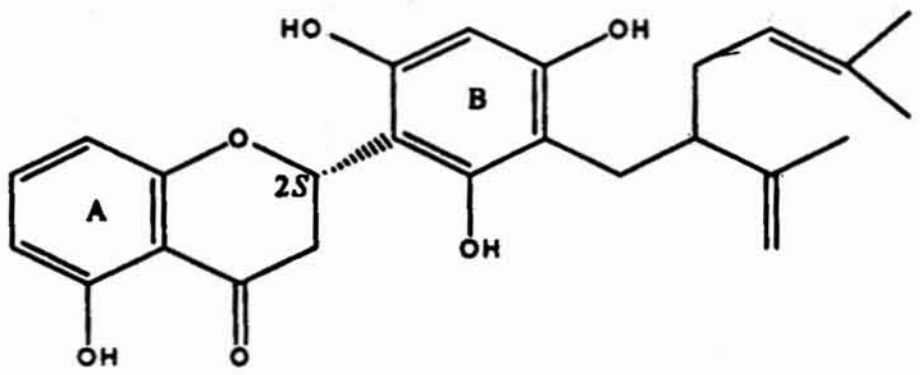

Fig. 2. Absolute configuration of **TW-1**

In order to prove the antioxidant capacity of this novel flavanone compound, the ability of selected compound to reduce the amount of peroxyl radicals (ORAC assay) was determined. The compounds' ability to scavenge radicals is expressed as Torlox equivalent, that is, their relative ability compared to the highly potent compound Trolox. Pure compound (**TW-1**) tested is highly antioxidant (2.25 Trolox equivalents) in comparison to the strong activity of natural polyphenols (Decker, *et al.*, 2008). In addition, novel compound (**TW-1**) was measured on its influence to its cytotoxic effect on HeLa human cervix carcinoma cells and Caco-2

human epithelial colorectal adenocarcinoma cells using the colorimetric methyl thiazoletetrazolium (MTT) assay. **TW-1** tested against HeLa cells showed an IC_{50} value between 4.6 and 5.5 μM, proving its moderate cytotoxic potential. Interestingly, it showed the lower cytotoxicity (IC_{50} value range 10.14-15.59 μM) on Caco-2 cells.

Conclusion

Novel flavanone type compound (**TW-1**) was isolated from the ethyl acetate extracts of stem of *Hypericum calycinum* L. (Myanmar name: Pyin-Nyar-Lin-Kar) for the first time. The structure of this compound was elucidated on the basis of 1D and 2D-NMR spectroscopic analysis as well as on their mass spectra. In order to learn about the stereochemistry of these compounds (at the C-2 position), CD (Circular dichroism) spectra are performed. In addition, cytotoxic effect of pure compound (**TW-1**) against HeLa human cervix carcinoma cells and Caco-2 human epithelial colorectal adenocarcinoma cells were determined using the colorimetric methylthiazoletetrazolium (MTT) assay. According to the results, the IC_{50} values (between 4.6 and 5.5 μM) of **TW-1** on HeLa cells are lower than that of Caco-2 cells. It can be concluded that **TW-1** is not a high toxic compound. An anti-oxidant activity of this compound (**TW-1**) was evaluated by applying ORAC (Oxygen Radical Absorbance Capacity) assay. **TW-1** shows higher oxidant activity than Trolox (2.25 Trolox equivalent). It is a novel lead anti-oxidant. The present research work suggests that the stem of *Hypericum calycinum* L. might be a potential source of natural antioxidant and it can be useful as in the formulation for the treatment of diseases such as cardiovascular diseases, atherosclerosis, tumour, cancer, ageing related diseases, diseases of oxidative stress etc.

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