Elucidation of Bioactivity of Tricin Released by Thioacidolysis from Wheat Straw Lignin

Yunbo Zhao,[a] Junyi Zhou,[a] Hujun Niu,[a] and Yimin Xie [a,b,*]

Tricin is a complex compound with chemical bonds to phenylpropane units of lignin in gramineous plants, and it is predominantly bound to lignin by β-O-4 ether bonds. Thioacidolysis cleaves the alkyl aryl ether bonds, which releases tricin from the tricin-lignin complex and maintains the natural structure of the tricin. In this study, milled wood lignin (MWL) was isolated from wheat straw by Björkman's method, and the MWL was subjected to thioacidolysis to release tricin from the MWL. Medium-pressure preparative liquid chromatography was used for further purification. FT-IR and ^{1}H-NMR analyses showed that the purified fraction was composed mainly of tricin-type flavonoids. The extracted tricin had strong scavenging capacity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, with a half maximal inhibitory concentration (IC_{50}) of 51.9 mg/L. Drug sensitivity paper testing showed that the extracted tricin inhibited the growth of *Escherichia coli*. The bacteriostatic circle diameters of tricin from wheat straw MWL and quercetin standard were 9.17 and 7.52 mm, respectively. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that the tricin from wheat straw MWL had possible inhibitory effect on lung cancer A549 cells, with a maximum inhibitory performance of 44.5%.

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Keywords: Tricin; MWL; Thioacidolysis; Radicals scavenging; Antibacterial; Anticancer

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INTRODUCTION

Lignocellulosic biomass, including cellulose, hemicellulose, and lignin, is used widely in biorefinery and functional materials (Zhu *et al.* 2016). In addition to biomass macromolecules, plants contain flavonoids, alkaloids, phenylpropanoids, and other substances with low content (Peanparkdee and Iwamoto 2019). Flavonoids have unique biological activities and potential use in disease treatment. Tricin (5,7-dihydroxy-2-(4-hydroxy-3,5-dimethoxy-phenyl)-4H-chromen-4-one) is a physiologically active flavonoid found in monocotyledonous plants, and it shows specific functions in terms of physiological activities (Martens and Mithofer 2005). Tricin has a flavanone backbone with two phenyl rings and a heterocyclic ring (Li *et al.* 2016). These are the benzoyl (A ring), cinnamoyl (B ring), and heterocyclic rings (C-ring), which enable tricin to bind to the lignin structural units in the form 3-β and β-O-4 ether bonds, with β-O-4 ether bonds predominating (Wenzig *et al.* 2005), as shown in Fig. 1.

Tricin is biosynthesized in secondary metabolism of plant cell walls, and mostly it is bonded with in lignin (Li *et al.* 2016). Additionally, tricin is fully compatible with the lignification process and is a real lignin monomer (Lan *et al.* 2015). Tricin can initiate the

lignin chain, and it has a close interaction with the lignin structure as the nucleation site for lignification in monocotyledonous plants (Lan *et al.* 2016; Li *et al.* 2017). Del Río *et al.* (2012, 2020) isolated milled wood lignin (MWL) from wheat straw and identified tricin when its structure was analyzed using reductive cleavage. It was found the tricin was contained in the cellulolytic enzyme lignin (CEL) by isolating it from wheat straw. You *et al.* (2013) isolated MWL and alkali lignin (AL) from stems and foliage of *Arundo donax* Linn and found that the foliage with higher condensed G units contained a great amount of tricin, and the linkage between lignin and tricin was stable under alkaline conditions.

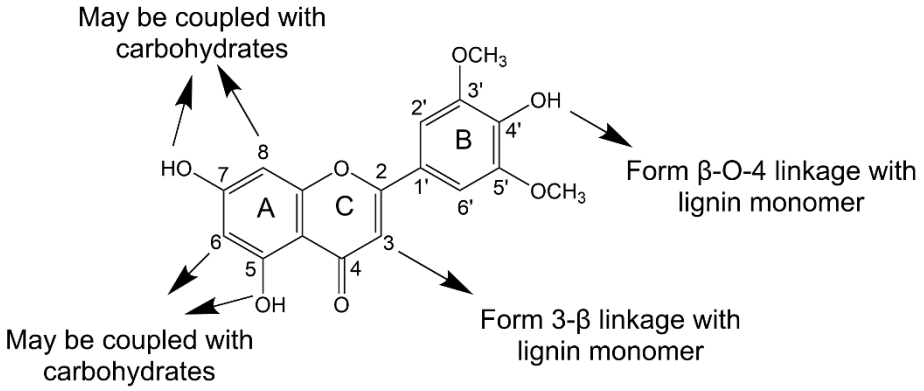

Fig. 1. Possible structure of tricin in wheat straw

Tricin has multiple functional groups, making it highly chemically reactive and physiologically active. These excellent biological properties endow them with the ability to inhibit the proliferation of certain viral cells (Chung *et al.* 2018), and they can be used as antioxidants, anti-inflammatory, and anticancer agents (Lee *et al.* 1981; Cui *et al.* 2011; Song *et al.* 2021). According to Zamora-Ros *et al.* (2017), tricin inhibits colon cancer cell proliferation, reduces mutations, and shields DNA damage *in vitro*. Tricin also induces phase I and phase II metabolic enzymes, changes cell growth signaling pathways, and mediates inflammation (Zamora-Ros *et al.* 2017). Tricin is also involved in the development and progression of pneumonia. Yang and Liu (2022) demonstrated that tricin could regulate AKT and MAPK signaling pathways to slow down lipopolysaccharide (LPS)-induced severe pneumonia by using ELISA and RT-qPCR to detect TNF-α, IL-1β and IL-6 levels. Ajitha *et al.* (2012) isolated tricin from *Njavara* rice bran and found that radical scavenging activity increased exponentially with an increase in the O-H bond dissociation enthalpy (BDE), by 90.39 μg/mL of tricin. Subsequent studies have found that tricin has a strong ability to scavenge ·OH radicals (Wang *et al.* 2020) and has an inhibitory effect on cancer (Yu *et al.* 2014; Song *et al.* 2022). Although tricin is a strong free radical scavenger, the tricin content in lignin was not necessarily higher, resulting in lower median effective concentration (EC_{50}) values for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (Xie *et al.* 2021). The current anticancer research on tricin is focused on rectal cancer, breast cancer, *etc.*, and its broad-spectrum anticancer activity has not been elucidated.

To obtain a high yield of tricin, lignin should be pretreated to cleave the covalent bond between tricin and other lignin moieties. Acid hydrolysis and reductive cleavage are commonly used to break the alkyl aryl ether bonds in lignin (Ikeda *et al.* 2002). Because tricin is recondensed with the lignin fragments, the degradation process greatly impacts the extraction of the tricin monomer. Thioacidolysis is a process that breaks the β-O-4 ether

bond on the chain of lignin, producing the lignin monomer and simultaneously releasing tricin. The reaction is catalyzed by BF_3 etherate and dioxane-ethanethiol (referred to as thioacidolysis solution). Thioacidolysis is an optimal method to both release tricin from the tricin-lignin complex and maintain the nature of the tricin. Thioacidolysis has been applied to the research on biosynthesis of lignin monomers and the bioprocessing of plant biomass (Kanazawa *et al.* 2009; Lapierre *et al.* 1999).

Understanding the structure and physiological characteristics of tricin will greatly promote the value-added application of polyphenol compounds in human health. In this study, tricin was isolated from wheat straw MWL with thioacidolysis, and the products were purified using medium-pressure preparative liquid chromatography, followed by structural characterization by FT-IR and ^{1}H-NMR. The free radical scavenging ability, antibacterial properties, and anticancer activities were also analyzed.

EXPERIMENTAL

Materials

Tricin standards were purchased from Chengdu Yirui Biotechnology Co., Ltd. (Chengdu, China). DPPH was purchased from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China) and Trolox was purchased from Shanghai Aladdin Biochemical Technology Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade. The Gram-negative bacterium *E. coli* ATCC 25922 was purchased from Shanghai Luwei Technology Co., Ltd. (Shanghai, China). The human A549 lung cancer cells were purchased from Beijing Beina Biotechnology Co., Ltd. (Beijing, China).

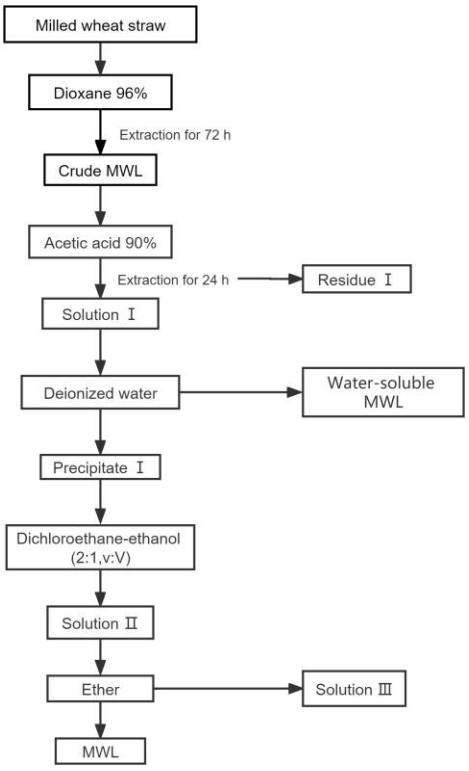

Fig. 2. Isolation of MWL from milled wheat straw

Isolation of Wheat Straw MWL

Wheat straw was air-dried, milled with a Wiley mill to a 40-60 mesh powder, and extracted with a benzene-ethanol mixture (2/1, v/v) in a Soxhlet extractor for 6 to 8 h. The milled straw was extracted using hot water. After drying with P_2O_5 *in vacuo*, the milled straw was further ground using a water-cooled vibratory ball mill for 72 h. The wheat straw MWL was extracted using Björkman's method (Björkman 1957), as shown in Fig. 2. MWL (0.40 g) was obtained from 20 g of ball-milled wheat straw powder, with a yield of 2.0%.

Thioacidolysis of Wheat Straw MWL

Dioxane, BF_3 etherate, and ethanethiol (40/1/20; v/v/v) were rapidly placed in an Erlenmeyer flask. Dioxane was used to determine the final volume of the solution. The thioacidolysis procedure is illustrated in Fig. 3. An appropriate amount of wheat straw MWL and thioacidolysis reagent were added to a dry Teflon tube, and the reaction mixture was heated to 100 °C for 8 h. The mixture was then transferred to a separation funnel, and then dichloromethane was added. After complete separation, 0.4 M $NaHCO_3$ was added to the upper aqueous phase to adjust the pH to 3 to 4, and then extracted twice with dichloromethane. The organic layer was evaporated under reduced pressure to obtain thioacidolysis products. The process of thioacidolysis is illustrated in Fig. 4.

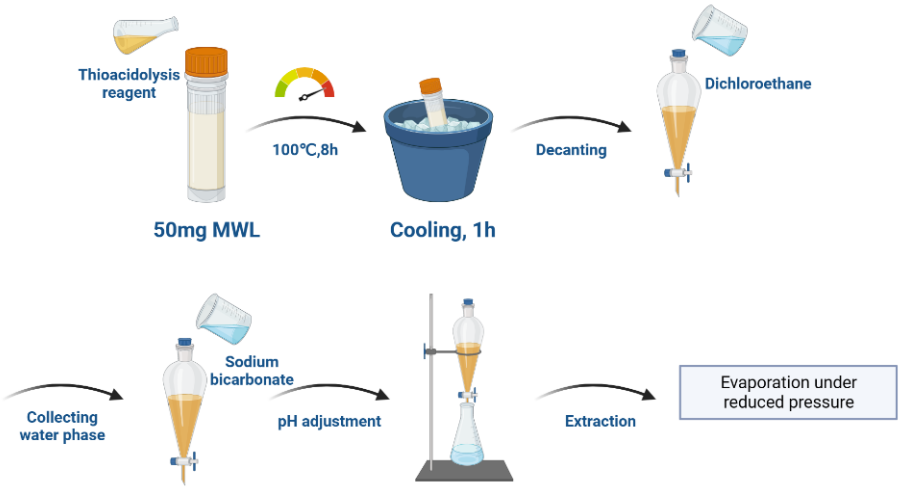

Fig. 3. Process of thioacidolysis of the MWL

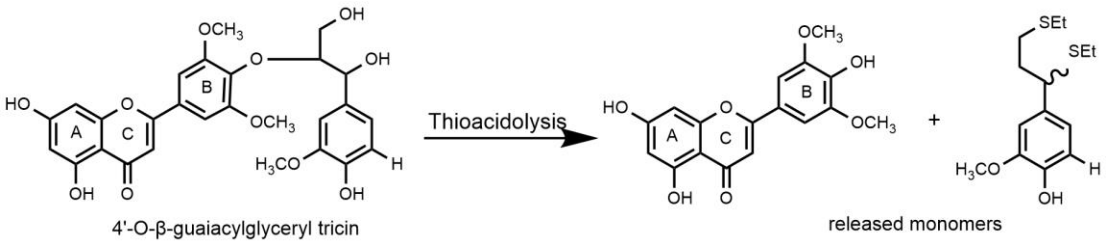

Fig. 4. Selective cleavage of ether bond in the MWL by thioacidolysis

Isolation of Tricin from Thioacidolysis Products

First, thioacidolysis products were dissolved in 80% methanol. After ultrasonic wave treatment and filtration through a 0.22 μm microporous membrane, the products were purified by a medium pressure preparative liquid chromatograph (Buchi C-615, Zurich, Switzerland). The chromatography parameters were as follows: column: BUCHI-CODE

NO.28139 (1.3 cm×46 cm) packed with 100-200 mesh Sephadex LH-20 dextran gel; detector: UV2302II UV- visible detector (Elite, 2-2 Xuezi Street, Dalian, China); detection wavelength: 330 nm; flow rate: 2.5 mL/min; mobile phase: 80% methanol. Two components were collected with retention times of 0 to 42 min and 42 to 78 min. The later component was concentrated under low temperature and reduced pressure, and 20 mg of the target product was obtained from 1.51 g of wheat straw MWL hydrolyzed with a yield of 1.3%.

Determination of FT-IR Spectrum of the Tricin from Thioacidolysis Products

A total of 3 mg of the sample was mixed with 150 mg of KBr. After grinding for 5 min, the mixture was pressed for 2.5 min at 18 MPa. The infrared spectra of the samples were measured using a Nicolet 6700 FT-IR (Thermo Fisher Scientific, Waltham, MA, USA) with a wavenumber range of 4000 to 500 cm^{-1} and a resolution of 2 cm^{-1}.

Determination of ^{1}H-NMR Spectroscopy of the Tricin Isolated from Thioacidolysis Products

The tricin product was dissolved in 0.5 mL of DMSO-d_6 and transferred to a Φ5 mm NMR tube. The corresponding ^{1}H-NMR spectroscopy was obtained by scanning the sample at 20 °C and 600.17 MHz using a JNM-ECZS series 600 MHz NMR spectrometer (Musashino, Akishima City, Tokyo, Japan). Settings included PD: 5.000s; AQ: 1.0905s; and scanning 64 times.

Determination of DPPH Radical Scavenging Capacity of the Tricin from Wheat Straw MWL

The 3 mL of 60 μM ethanol solution of DPPH was added to the appropriate amount of sample solution and stirred. After 5 min, the absorbance at 517 nm was determined using a UV-2250 spectrophotometer (Shimadzu, Nakagyo-Ku, Kyoto, Japan), with 95% ethanol as the reference. The inhibition performance was calculated with Eq. 1. The half maximal inhibitory concentration (IC_{50}) was calculated by fitting the curve using SPSS 26 software (Andrade *et al*. 2017; Das *et al*. 2022).

$$\text{Inhibition performance (IP\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \quad (1)$$

Determination of the Antibacterial Activity Against *E. Coli* of the Tricin Isolated from Wheat Straw MWL

Determination of bacterial concentration

The turbidity method of McFarland is a conventional method for determining the concentration of bacteria by comparing the turbidity of the bacterial suspension with a standard in a turbidity tube (McFarland 1907). The measurements were based on the different turbidities of precipitated barium sulfate obtained by reaction with different concentrations of $BaCl_2$ and H_2SO_4 solutions. The turbidity of the bacterial suspension was found to be equivalent to the turbidity of a corresponding standard by visual observation. The approximate concentration of bacterial suspension can be determined by using the different refraction of light in different concentrations of liquid.

A 0.25% $BaCl_2$ and 1% H_2SO_4 solution were prepared and mixed in six test tubes, numbered as shown in Table 1. The broth culture was added to a sterile test tube, and sterile saline was added to the test tube until the target concentration was reached.

Table 1. Ratios of Solutions for the McFarland Method

Test tube No.	1	2	3	4	5	6
0.25% $BaCl_2$ (mL)	0.2	0.4	0.8	1.2	1.6	2.0
1% H_2SO_4 (mL)	9.8	9.6	9.2	8.8	8.4	8.0
Proximate concentration of bacteria($\times 10^8$/mL)	1	3	6	9	12	15

Measurement of the inhibition zone

A 5.5 mm diameter filter paper disc was autoclaved, and 1 to 4 mg of plant extractive was dissolved in 4% DMSO absolute ethanol solution. The dry filter paper discs were immersed in the dissolved extractive for 5 h to prepare the drug-sensitive paper discs. The diluted bacterial suspension (0.1 mL) was uniformly applied to a sterile plate with the plate-coating method. Then 3 to 4 drug-sensitive paper discs of each extractive were pasted on the sterile medium, while a control group with filter paper discs were soaked in 4% DMSO ethanol solution and a blank group with filter paper discs were not soaked but with the solution applied. The above samples were cultured at 37 °C for 24 h, and the size of the inhibition zone was measured using a Vernier caliper (Xu *et al*. 2021).

Determination of Anticancer Activity of the Tricin Isolated from Wheat Straw MWL

Human lung cancer A549 cells were cultured, rinsed with PBS buffer, and digested using trypsin. RPMI-1640 complete medium was added to the blank and control wells, while the cell suspension was added to the sample well. After the cells were cultured in a CO_2 incubator, they completely adhered to the bottom layer. PBS buffer was added to the control well, and different concentrations of the tricin sample solution dissolved in PBS were added to the sample well. After 24 h of cultivation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the blank, control, and sample wells, and the culture was terminated after 4 h. DMSO was added after the culture solution had been absorbed into the wells. After shaking at low speed for 10 min, the absorbance at 490 nm was measured with a NanoQuant Infinite M200pro enzyme calibration (Seestrasse 103, 8708 Männedorf, Switzerland).

RESULTS AND DISCUSSION

Analysis of FT-IR Spectrum of the Tricin Isolated from Thioacidolysis Products

The FT-IR spectrum of the tricin from wheat straw MWL is shown in Fig. 5. Most of the moieties in the standard tricin products could be observed in the tricin from the thioacidolysis products. Absorbance due to the benzene ring was at 1510 to 1610 cm^{-1}, indicating the presence of A-ring and B-ring structures of the tricin in the thioacidolysis products of the MWL. A conjugated carbonyl group, *i.e.*, ketone group, was observed at 1646 to 1660 cm^{-1}, indicating the presence of a C-ring. The stretching vibrations at 3411 to 3446 and 2850 to 2927 cm^{-1} correspond to the hydroxyl, methyl, methylene, and methylidyne groups, respectively. These findings are in accord with those from the infrared spectrum of the tricin standard, supporting the presence of the tricin in the thioacidolysis products of wheat straw MWL.

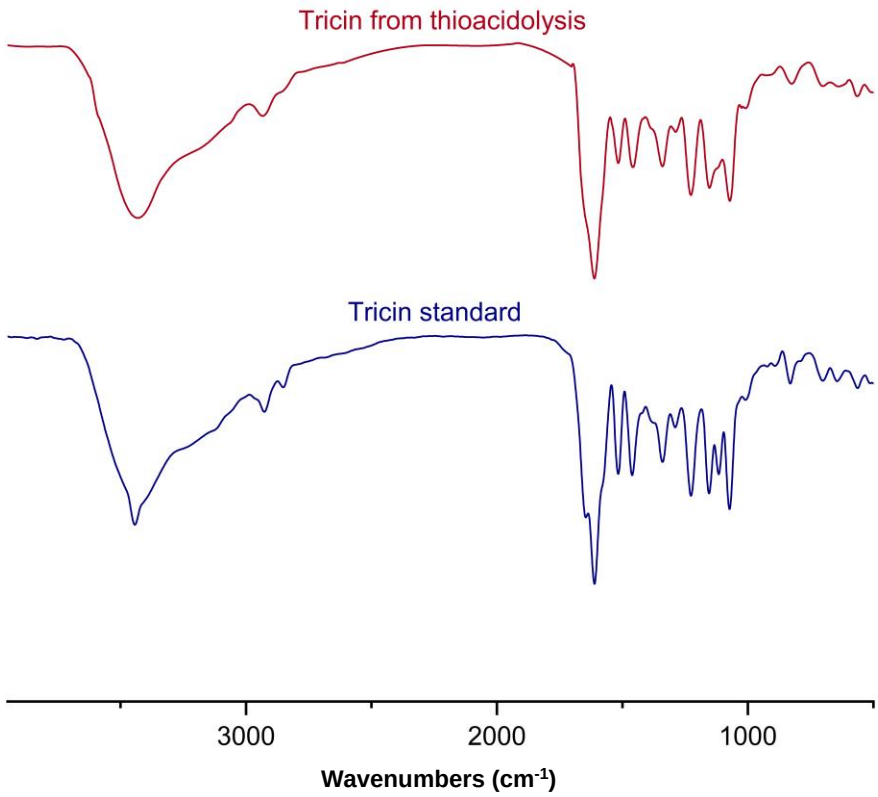

Fig. 5. FT-IR of the tricin from thioacidolysis and tricin standard

Analysis of ^{1}H-NMR Spectrum of the Tricin Isolated from Thioacidolysis Products

Two separated fractions with different retention time were collected by medium pressure liquid chromatography, 0 to 42 min and 42 to 78 min, respectively. The 0 to 42 min fraction did not contain tricin. It likely contained derivatives of lignin monomer from thioacidolysis. The ^{1}H-NMR spectrum of the 42 to 78 min fraction of the thioacidolysis products of wheat straw MWL is shown in Fig. 6. The signal assignments are listed in Table 2. The signals at δ 12.93 ppm (5-OH), δ 7.29 ppm (H2′, H6′), δ 6.95 ppm (H3), δ 6.53 ppm (H8), δ 6.17 ppm (H6), δ 3.84 ppm (3′, 5′ -OCH_3) are from aromatic rings, which indicates that the structure of the tricin was isolated from wheat straw MWL (National Center for Biotechnology Information, 2023). The signals at δ H:1.02 (CH_3), 1.08 (CH_3), 1.13 (CH_3), 1.18 ppm (CH_3), 2.42 ppm (CH_2). 2.53 ppm (CH_2), 2.76 ppm (CH_2), 2.78 ppm (CH_2), 2.99 ppm (CH_2), 3.13 ppm (C_β), 3.34 ppm (H_2O), 3.71 ppm (C_β), and 4.18 ppm (C_α), are from aliphatic side chains (Jiao *et al.* 2007; Yue *et al.* 2012). This means that it contained some impurities.

Considering the similarity between FT-IR spectrum of this product and that of standard tricin, the impurity content can be estimated to be less than 5%. A signal at δ H3.84 ppm (3', 5' -OCH_3) was strong, indicating the presence of more methoxy groups in the fraction. It is possible that some lignin moieties with a β-O-4 linkage to the syringyl or guaiacyl were also degraded during thioacidolysis, resulting in the high methoxy content of the fraction.

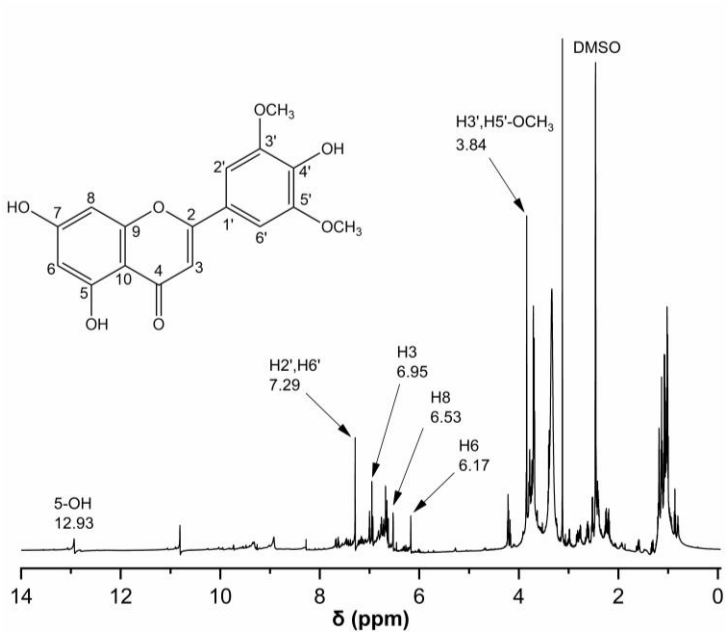

Fig. 6. ^{1}H-NMR spectrum of the tricin thioacidolysis products

Table 2. Assignment of the Signals in ^{1}H-NMR Spectrum of the Tricin Isolated from Thioacidolysis Products

δH (ppm)	Assignments
12.93	5-OH
7.29	H2', H6'
6.95	H3
6.53	H8
6.17	H6
4.18	H-C_α
3.71, 3.13	H-C_β
3.84	3', 5'-OCH_3
3.34	H_2O
2.99, 2.78, 2.53, 2.42	CH_2
2.46	DMSO
1.18, 1.13, 1.08, 1.02	CH_3

Analysis of DPPH Free Radical Scavenging Characteristic of the Tricin Isolated from Wheat Straw MWL

The radical-scavenging effects of quercetin standard and the tricin from wheat straw MWL, using the DPPH assay and software analysis, are shown in Fig. 7. The IC_{50} values were 45.1 and 51.9 mg/L, respectively (Table 3). The C2 = C3 bond on the C-ring can couple with the electron delocalized on the B-ring and join to the ketone group on the C-ring, providing a stronger stabilizing effect on DPPH free radicals. Hydroxyl groups at 5, 7, and 4′ positions in tricin chelate with transition metals, stabilizing the free radicals and enhancing the radical scavenging capacity of the tricin. Compared with quercetin standards, the tricin in wheat straw MWL required higher concentrations to achieve the same scavenging effect on DPPH free radicals due to the presence of methoxy groups. The methoxy groups replace the hydroxyl groups, weakening the radical scavenging capacity of tricin. However, the IC_{50} of the tricin from wheat straw MWL was still lower than that of most lignocellulosic biomass (Gharibi *et al*. 2015; Tohidi *et al*. 2017).

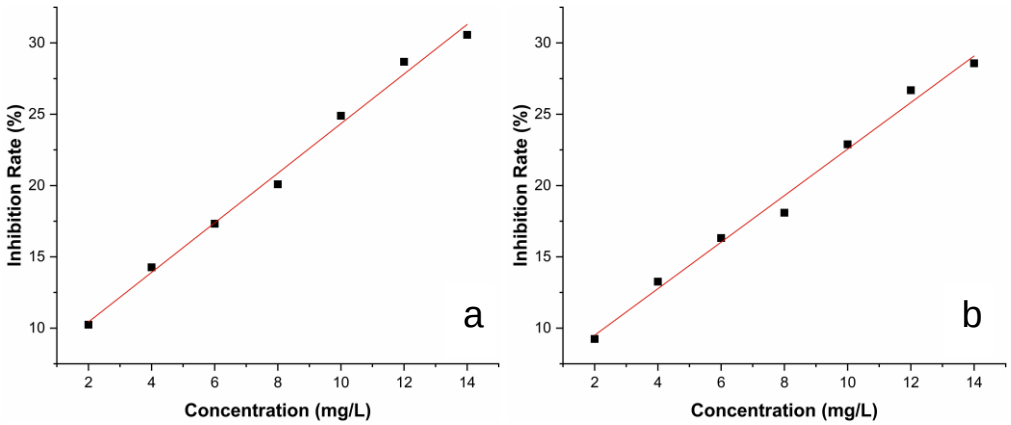

Fig. 7. Radicals scavenging effect of quercetin standard (a) and the tricin from wheat straw MWL (b) on DPPH

Table 3. Free Radicals Scavenging Effect of the Tricin from Wheat Straw MWL and Quercetin Standard on DPPH

Compounds	IC_{50} (mg/L)
Quercetin standard	45.1
Tricin of wheat straw MWL	51.9

Analysis of Antibacterial Characteristics of the Tricin Isolated from Wheat Straw MWL

The inhibitory effect of the tricin from the wheat straw MWL on *E. coli* was investigated with a drug-sensitive paper method, with a paper diameter of 5.5 mm (Fig. 8).

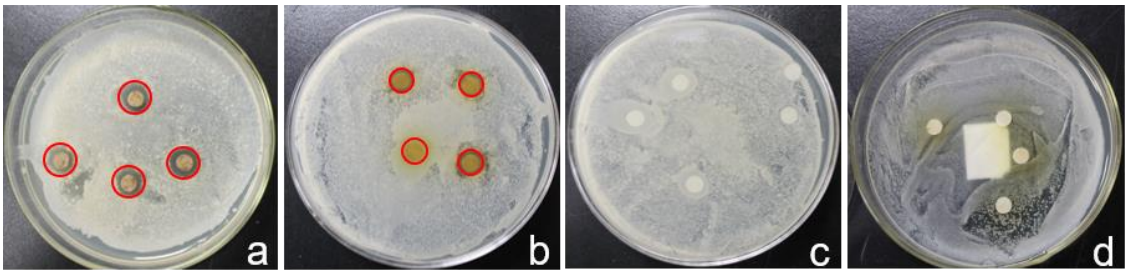

Fig. 8. Inhibition effects of the tricin from wheat straw MWL, and quercetin on *E. coli*
a: tricin from wheat straw MWL, b: quercetin, c: control, d: blank

The inhibition zone diameters of the tricin from wheat straw MWL and quercetin standard were 9.17 mm and 7.52 mm, respectively. However, there was no inhibition zone around the drug-sensitive paper of 4% DMSO ethanol solution in the control group, indicating that the tricin from wheat straw MWL had the strongest inhibitory effect on growth of *E. coli*. The tricin from wheat straw MWL mainly inhibits the synthesis of gene nucleic acids in bacteria, changing cell membrane permeability and inhibiting cell membrane porin to achieve antibacterial effects (Xie *et al*. 2015). Porin is a doughnut-shaped transmembrane protein that spans a membrane in living cells to create a channel for the passage of small molecules. It is found in the outer membrane of mitochondria as well as in the outer membrane of gram-negative bacteria. Hydroxyl groups at the 5, 7, and 4'

positions of tricin can also increase antibacterial activity. The C-ring is an oxygen-containing heterocyclic group that moderately improves the antibacterial activity of tricin. The ^{1}H-NMR spectrum of the tricin from wheat straw MWL showed that the tricin contains methyl, methylene, and aliphatic impurities. If further purified, the products may have a stronger inhibitory effect on *E. coli*.

Analysis of Anticancer Characteristics of the Tricin Isolated from Wheat Straw MWL

Inhibition performances of the tricin isolated from wheat straw MWL on lung cancer A549 cells are nonlinearly fitted in Fig. 9. At the beginning, increased concentration of tricin enhanced the inhibition on lung cancer A549 cells. When the concentration of tricin was 70.77 μg/mL, the inhibition was maximal at 44.52%. However, as concentration of the tricin was further increased, inhibition performance decreased. These results indicate that the isolated tricin may contain sugar impurities. Therefore, tricin inhibits the proliferation of lung cancer cells at relatively low concentrations. As the concentration of tricin increased, the concentration of sugar impurities also increased. The effect of sugars on cell growth was stronger than that of the tricin on cell growth inhibition. In general, tricin exerted an inhibitory effect on lung cancer A549 cells. However, further purification and clinical trials are needed for its medical use as an anticancer drug.

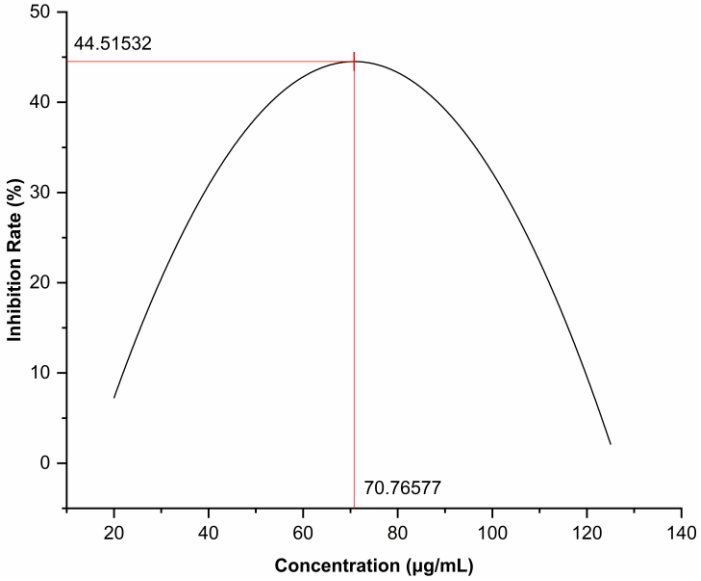

Fig. 9. Inhibitory effect of tricin on lung cancer A549 cells

CONCLUSIONS

1. After isolation of milled wood lignin (MWL) from wheat straw using Björkman's method, the β-O-4 ether bond was selectively broken by thioacidolysis to release tricin. ^{1}H-NMR results of the 42 to 78 min fraction obtained by medium-pressure preparation liquid chromatograph showed chemical shifts of δ 12.93 ppm (5-OH), δ 7.29 ppm (H2', 6'), δ 6.95 ppm (H3), δ 6.53 (H8), δ 6.17 ppm (H6), and δ 3.84 ppm (3', 5' -OCH_3), indicating the structure of the tricin isolated from wheat straw MWL. However, the product contains some impurities of aliphatic substances.

2. The free radical scavenging ability of the isolated tricin against DPPH free radicals was investigated using UV-visible spectrophotometry. The tricin in the free state had a strong scavenging ability on DPPH free radicals. The IC_{50} value was 51.9 mg/L, which was attributable to the coupling of C2 = C3 on the C-ring with the electrons delocalized on the B-ring joining to the ketone group on the C-ring. Hydroxyl groups at 5, 7, and 4′ positions in the tricin can also stabilize free radicals, making the tricin from wheat straw MWL highly resistant to oxidation.
3. Use of the drug sensitivity paper method indicated that the tricin from wheat straw MWL inhibited growth of *E. coli*. Inhibition circle diameters from the isolated tricin and quercetin standard were 9.17 and 7.52 mm, respectively. The stronger inhibitory effect of the isolated tricin was mainly due to changes in cell permeability and inhibition of cell membrane pore protein resulting from inhibition of gene nucleic acids in bacteria.
4. The inhibitory effect of the tricin isolated from wheat straw MWL on lung cancer A549 cells was investigated with the MTT method. The maximum inhibition performance of the tricin on lung cancer A549 cells was 44.5%, indicating that the isolated tricin moderately inhibits the growth of lung cancer A549 cells. However, it cannot be concluded that tricin can be utilized in the medical field as an anticancer drug.

ACKNOWLEDGMENTS

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