

PREDICTION OF *trans*-CINNAMIC ACID IN SINYO NAKAL (*Duranta repens*) FRUITS' METHANOL EXTRACT

ABSTRACT

Aims: There are many reports about chemical compounds in leaves and in whole plants of *sinyo nakal* (*Duranta repens*). However, only a few study regarding the chemical components of its fruits, especially on *D. repens* grown in Indonesia. In this study, qualitative phytochemical tests showed the presence of tannins, saponins, alkaloids, flavonoids, and steroids in crude methanol extract of *D. repens* fruit from Jombang, East Java.

Study design: Experimental study

Place and Duration of Study: Organic Chemistry Laboratory, Chemistry Department, Mathematics and Sciences Faculty Bogor Agriculture University, and Bandung Technology Institute, between February and December 2013.

Methodology: This fruit has been extracted with *n*-hexane in previous study to remove the nonpolar components. Fractionation of the methanol extract was done by using step gradient elution from *n*-hexane to ethyl acetate, followed by methanol.

Results: Methanol fraction gave the highest yield. A yellowish white crystal was obtained in further fractionation of this fraction, which is predicted as crude *trans*-cinnamic acid based on nuclear magnetic resonance spectra analysis. The compound is still impure and can not be purified and characterized further because of limited amount of sample.

ABSTRACT

There are many reports about chemical compounds in leaves and in whole plants of *sinyo nakal* (*Duranta repens*). However, only a few study regarding the chemical components of its fruits, especially on *D. repens* grown in Indonesia. In this study, qualitative phytochemical tests showed the presence of tannins, saponins, alkaloids, flavonoids, and steroids in crude methanol extract of *D. repens* fruit from Jombang, East Java. This fruit has been extracted with *n*-hexane in previous study to remove the nonpolar components. Fractionation of the methanol extract was done by using step gradient elution from *n*-hexane to ethyl acetate, followed by methanol. Methanol fraction gave the highest yield. A yellowish white crystal was obtained in further fractionation of this fraction, which is predicted as crude *trans*-cinnamic acid based on nuclear magnetic resonance spectra analysis. The compound is still impure and can not be purified and characterized further because of limited amount of sample.

Keywords: *trans*-cinnamic acid, *Duranta repens*, fruits methanol extract

1. INTRODUCTION

Duranta repens is a member of *Duranta* species which is widely spread in tropical and subtropical regions [1]. Most of this plants are cultivated as ornamental plants in West India, Pakistan, Middle and South America. In Indonesia, *D. repens* is generally known as *sinyo nakal*. Its fruits are traditionally used to treat malaria and worms, while its leaves are used as abscess medicine, antipyretic, diuretic, and antimalarial agents [2].

Several studies concerning the bioactivities of *D. repens* have been reported. The fruits and stem show antifungal and antibacterial activities, cytotoxicity, and larvicidal activity against *Culex quinquefasciatus* [3, 4]. The leaves show antibacterial [2], antioxidant [5], and larvicidal activities against *C. quinquefasciatus* [6]. The whole plant also shows antioxidant, antiviral [7], and insecticidal activities against *Aedes aegypti* and *Attagenus piceus* [8], as well as α -glucosidase enzyme inhibitors [9].

Many active compounds have been isolated from the leaves and the whole plants of *D. repens*, including terpenoids and steroids [1, 3, 4, 7, 10–14], flavonoids [1, 7, 9, 12], iridiod glycosides [10], coumarins and coumarinolignoids [8, 13], and phenylethanoid glycosides [1, 2]. Still few studies on *D. repens* fruits have been conducted. Moreover, different growing place can influence the amount and type of secondary metabolites produced by a plant. Therefore, this study aims to characterize the chemical constituents of *D. repens* fruits from Indonesia. The isolation and characterization are focused on the polar fraction of the methanol extract.

2. MATERIAL AND METHODS

2.1 Materials

Materials used include *D. repens* (collected from Jombang, East Java); twice distilled technical grade methanol, ethyl acetate, *n*-hexane, and dichloromethane; Wagner, Mayer, Dragendorf, and Liebermann-Buchard reagents; 10% NaOH (technical); 1% FeCl₃ (technical); H₂SO₄ (p.a); silica gel Merck 60 G for thin layer chromatography (TLC); silica gel Merck 60 (0.2–0.5 mm and 0.063–0.200 mm) for column chromatography; and silica gel Merck 60 GF₂₅₄ for TLC.

2.2 Instrumentation

Basic instruments used were TLC plates (silica gel 60 F₂₅₄); liquid vacuum chromatography (LVC), gravitational column chromatography, and radial chromatography apparatus; rotary evaporator; and a set of common glassware. The nuclear magnetic resonance (NMR) spectra were obtained with an Agilent spectrometer working at a frequency of 500 MHz (¹H) and 125 MHz (¹³C) at Bandung Institute of Technology.

2.3 Preparation and Extraction

Milled dried sample (2.28 kg) containing 12.0% of water was macerated in *n*-hexane for 24 hours. The filtrate was then saponified by liquid-liquid extraction with several portions of 0.5 N NaOH. The unsaponified *n*-hexane extract (the upper phase) was collected and evaporated under reduced pressure. The sample residue, which is now free of lipid and other non-polar components, was re-macerated in methanol (6 L) for 24 hours. This maceration was done in three replications. Phytochemical tests were conducted on the *n*-hexane and methanol extract based on standard procedures [15].

2.4 Secondary Metabolites Compounds Isolation from Methanol Extract

The first fractionation step was tannin removal. Tannin was precipitated by pouring acetone (100 mL) into the methanol extract, and the precipitate was separated by filtration. This step was repeated until virtually no more precipitate was formed. The tannin-free methanol extract was then concentrated under reduced pressure.

Best eluent for fractionation of the extract was then determined. TLC plate (silica gel 60 F₂₅₄) was used as the stationary phase. Various eluents, from nonpolar to polar (*n*-hexane, dichloromethane, chloroform, ethyl acetate, acetone, and methanol, respectively), was tested. Two eluents resulting the most well-separated spots on the TLC plate were combined in several compositions to obtain the best eluent composition.

Brief fractionation was done by using LVC. Silica gel Merck 60 G for TLC was used as the stationary phase. The extract was pre-absorbed in silica gel Merck 60 (0.2–0.5 mm) for column chromatography. Step gradient elution with increasing polarity was then carried out by using combination of two eluents, one of which retarded the spots near the start line of the TLC plate, whereas the other one ran the spots near to the finish line. The separation pattern of the eluates was checked by using the best eluent. Eluates with same separation pattern were combined into fractions.

Fraction with the highest yield was fractionated further by using gravitational column chromatography with the same step gradient elution system. Silica gel Merck 60 (0.063–0.200 mm) was used as the stationary phase. Fractions which gave single spot on the TLC plate were elucidated by using NMR spectrometer. Fractions containing a few components was purified further by using radial chromatography before being elucidated. Silica gel Merck 60 GF₂₅₄ for TLC was used as the stationary phase. The best eluent was determined with the same procedure as above, and used as the mobile phase in isocratic elution system.

3. RESULTS AND DISCUSSION

From 2.28 kg of *D. repens* fruits, 45.2 g of *n*-hexane extract was obtained, 10.0 g of which was unsaponifiable. Maceration of the residue in methanol resulted 466 g of methanol extract, or 23.2% of yield based on dried weight (12.0% of water content). Based on phytochemical studies, the unsaponifiable *n*-hexane extract only contains steroids, whereas the methanol extract also contains tannins, saponins, alkaloids, and flavonoids. Steroids were evidenced by green colour formation in Liebermann-Burchard test. Tannins produced blue colour with 1% FeCl₃. Saponins formed stable foam after being shaken. Alkaloids were proved by orange, yellowish white, and brown precipitate with Dragendorff, Mayer, and Wagner reagents, respectively. Flavonoids produced red colour with 10% NaOH and pink colour with concentrated H₂SO₄. Jayalaksmi *et al.* [2] reported that *D. repens* leaves' methanol extract contains tannins, saponins, flavonoids, steroids, and terpenoids. Serena *et al.* [6] reported tannins, saponins, alkaloids, flavonoids, and terpenoids in the same extract. These results show that both *D. repens* leaves and fruits contain approximately the same group of compounds, except terpenoids, which is only found in *D. repens* leaves and not in its fruits.

Tannin removal from 49.1 g of the methanol extract left 28.1 g of tannin-free methanol extract. This extract is soluble in acetone. The best eluent composition for this extract is *n*-hexane-ethyl acetate (4:6). Step gradient elution in LVC apparatus was carried out on this extract (20.0 g) by using *n*-hexane (1×), *n*-hexane-ethyl acetate 8:2 (2×), 6:4 (2×), 5:5 (4×), 4:6 (2×), 3:7 (2×), ethyl acetate (2×), and methanol (10×), respectively. Nine fractions are collected based on TLC checking using the best eluent (Table 1).

Table 1 LVC fractions from tannin-free methanol extract of *D. repens* fruit

Fraction	Eluent	Weight (g)	Yield (%)
A	<i>n</i> -Hexane + <i>n</i> -hexane-ethyl acetate 8:2 (elution 1)	0.0105	0.05
	<i>n</i> -Hexane-ethyl acetate:		
B	8:2 (elution 2) + 6:4 (elution 1)	0.113	0.57
C	6:4 (elution 2) + 5:5 (elution 1)	0.194	0.97

D	5:5 (elution 2–4)	0.289	1.44
E	4:6	0.089	0.44
F	3:7	0.115	0.57
G	Ethyl acetate	0.224	1.12
H	Methanol (elution 1–2)	12.5	62.4
I	Methanol (elution 3–10)	4.82	24.1

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Fraction-H has the highest yield (62.4%). This fraction is eluted in methanol. Tannins were still detected in this fraction and removed by precipitation in acetone to avoid spot tailing during further fractionation. From 12.5 g of fraction-H, 6.45 g of tannin-free fraction-H was obtained, and 3.95 g of which was fractionated by using a gravitational column chromatography. Step gradient elution system of *n*-hexane-ethyl acetate gives 15 fractions. Two fractions (H₆ and H₉) produce only one spot each in the TLC test, with *R_f* value of 0.83 and 0.65, respectively, using the best eluent. Fraction H₆ was obtained only in small amounts (1.7 mg), so that only fraction H₉ (13.3 mg) was elucidated with ¹H NMR. However, its spectrum still shows mixture of compounds and can not yet be determined.

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The most abundant fraction, H₇ (51.05 mg), produce only two components in TLC test with *R_f* value of 0.83 and 0.60. These components was then purified by using radial chromatography. The best eluent composition is dichloromethane-ethyl acetate (3:7), The first fraction, H₇₁ (11.4 mg), is a yellowish white crystal with the same *R_f* value as fraction H₆. Its chemical structure was then elucidated from the NMR spectra.

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¹³C NMR spectrum (Fig. 1) shows 7 carbon signals from 9 carbon atoms in 117.2, 128.3, 128.9, 130.7, 134.0, 146.9, and 171.7 ppm. Signal at 171.7 ppm shows carbonyl group from carboxylic acid. Two signals at 117.2 and 146.9 ppm are *sp*² carbon signals with different chemical environment. The existence of benzene aromatic ring is proved by 4 signals in 128.3, 128.9, 130.7, and 134.0 ppm. Each of two signals in 128.3 and 128.9 ppm rises from two equivalent carbon atoms, based on their higher intensities than the two other signals. This chemical shift pattern matches with a monosubstituted benzene structural unit.

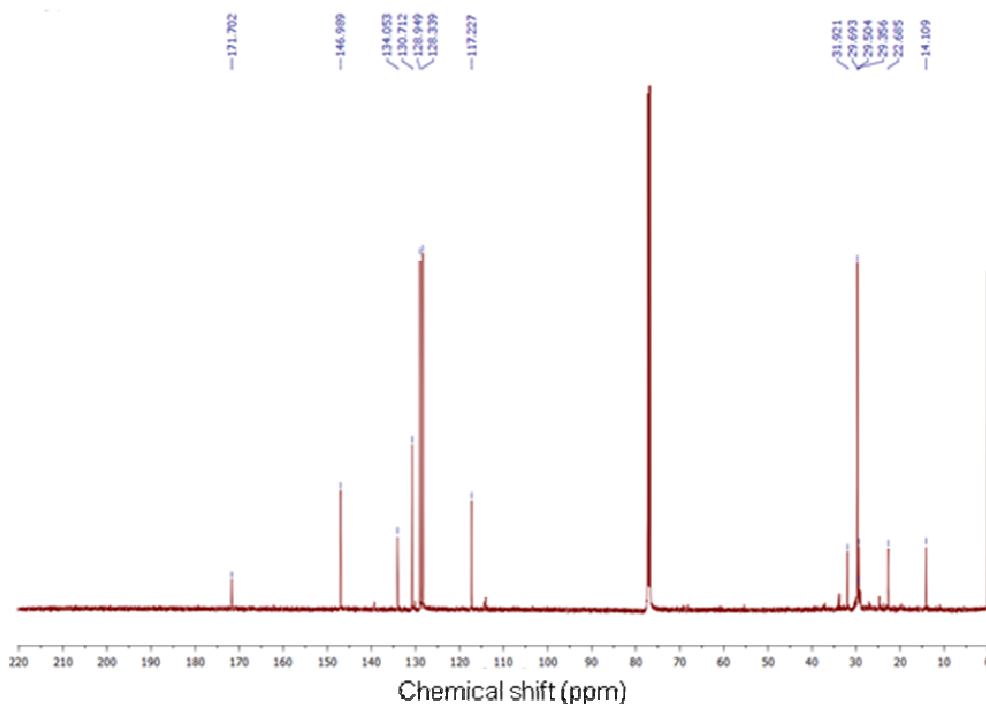


Figure 1 ^{13}C NMR spectrum of H_{71} fraction (125 MHz, CDCl_3)

^1H NMR spectrum (Fig. 2) shows 4 proton signals in 6.4, 7.4, 7.6, and 7.8 ppm. Signals in 6.4 ppm (1H, *d*) and 7.8 ppm (1H, *d*) show a *trans*-alkene group with coupling constant of 16 Hz. This result is supported by the total correlation spectroscopy (TOCSY) spectrum. When hydrogen in 6.4 ppm is irradiated, only peak in 7.8 ppm appears. It suggests a direct correlation between the two adjacent protons. One signal is more downfield (δ_{H} 7.8 ppm) because of deshielding effect. The double bond will resonate due to the electron-withdrawing effect of the carbonyl group leaving partial positive charge at the β -carbon. The α -carbon, which is not deshielded gives lower chemical shift. Aromatic protons of the monosubstituted benzene are evidenced from the absorption peaks in 7.4 ppm (3H, *t*) and 7.6 ppm (2H, *m*). However, the exchangeable proton of $-\text{COOH}$ does not appear in the ^1H NMR spectrum. It is possibly because of a very rapid proton exchange between the acidic proton and protons from water residue which is still left during the preparation of sample. The summary of ^1H and ^{13}C NMR analysis and their comparison with the literature [16] are shown in Table 2 and 3.

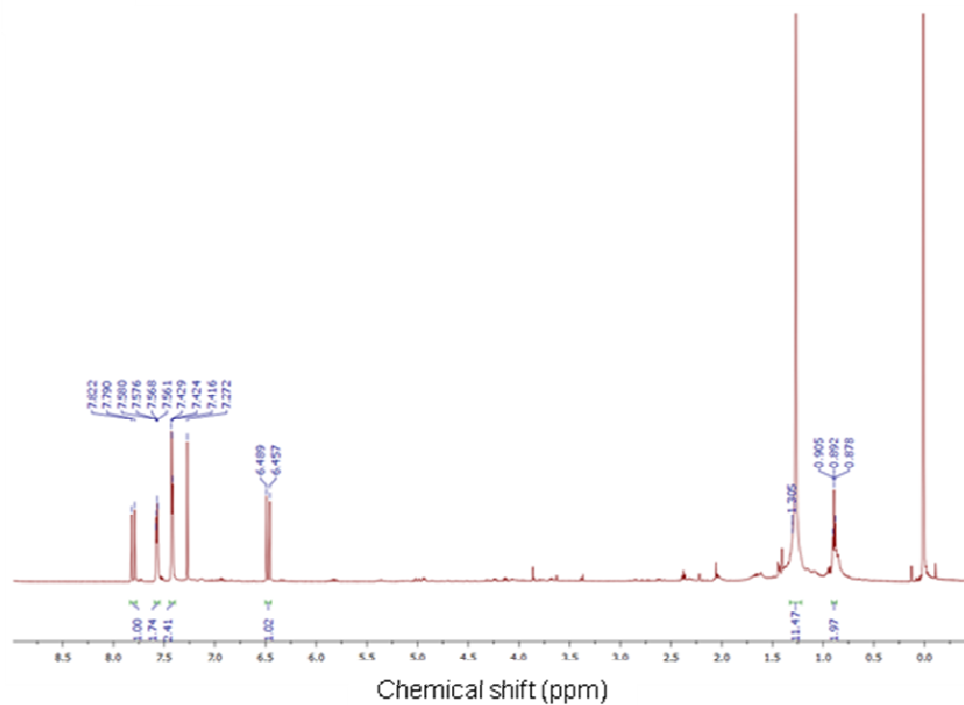
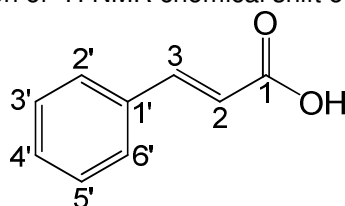


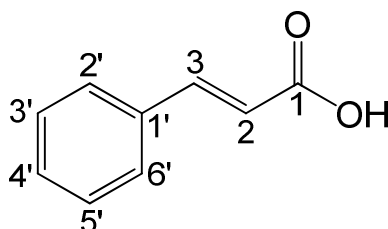
Figure 2 ^1H NMR spectrum of H_{71} fraction (500 MHz, CDCl_3)

Table 2 Comparison of ^1H NMR chemical shift of *trans*-cinnamic acid



H atom	This study (500 MHz, CDCl_3)		[16] (300 MHz, CDCl_3)	
	Σ H	δ_{H} (ppm)	Σ H	δ_{H} (ppm)
		(multiplicity, J (Hz))		(multiplicity, J (Hz))
OH	-	-	-	-
2	1	6.4 (<i>d</i> , 16)	1	6.48 (<i>d</i> , 15.9)
3	1	7.8 (<i>d</i> , 16)	1	7.82 (<i>d</i> , 15.9)
2'/6'	2	7.6 (<i>m</i>)	2	7.65–7.54 (<i>m</i>)
3'/4'/5'	3	7.4 (<i>t</i>)	3	7.5–7.36 (<i>m</i>)

Table 3 Comparison of ^{13}C NMR chemical shift of *trans*-cinnamic acid



C atom	δ_{C} (ppm) (multiplicity, J (Hz))	
	This study (125 MHz, CDCl_3)	[16] (75 MHz, $\text{DMSO}-d_6$)
1	171.7	167.66
2	117.2	119.35
3	146.9	144.01
1'	134.0	134.32
2'/6'	128.3	128.24
3'/5'	128.9	128.96
4'	130.7	130.26

The heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond coherence (HMBC) spectra give further evidence of the *trans*-cinnamic acid structure. The HSQC spectrum shows correlation between proton and carbon through a single bond. Signal correlations between δ_{C} 117.2 ppm and δ_{H} 6.4 ppm and also δ_{C} 146.9 ppm and δ_{H} 7.8 ppm prove the existence of a disubstituted alkenes, $\text{RCH}=\text{CHR}'$ (Fig. 3). The evidence of a monosubstituted benzene is explained as follows. Two *ortho* protons in δ_{H} 7.6 ppm are correlated with δ_{C} 128.3 ppm, two *meta* protons in δ_{H} 7.4 ppm are correlated with δ_{C} 128.9 ppm, and a *para* proton in δ_{H} 7.4 ppm is correlated with δ_{C} 130.7 ppm. The carbon signal in δ_{C} 134.0 ppm is not correlated with any signal in ^1H NMR spectrum, suggesting that it is a quaternary *ipso* carbon.

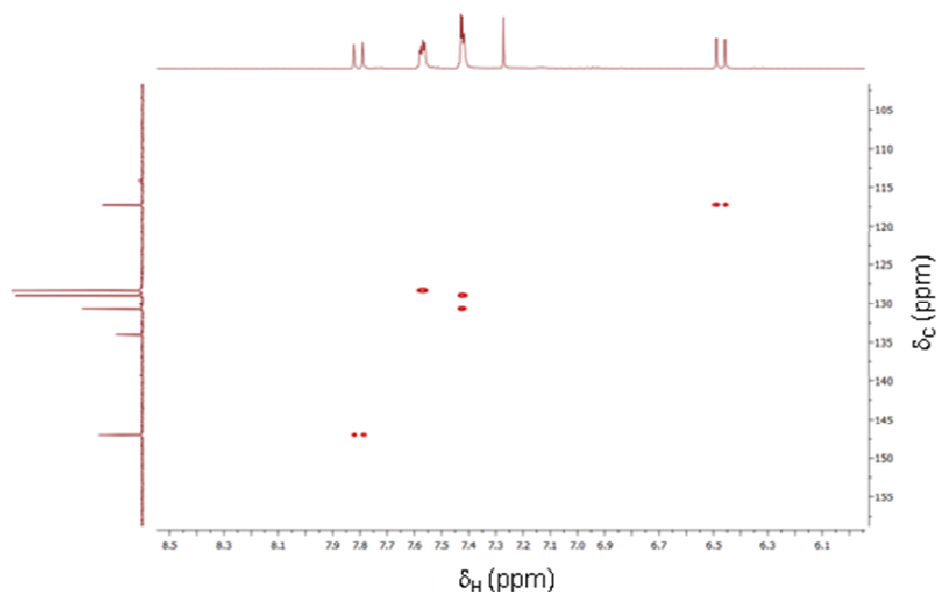


Figure 3 HSQC spectrum of H_{71} fraction

The HMBC spectrum shows correlation between proton and carbon atoms separated by two or three bonds. Conjugation of the carbonyl group with the double bond is evidenced from correlation between carbonyl signal (δ_C 171.7 ppm) and 2 proton signals (δ_H 6.4 and 7.8 ppm) (Fig. 4). The C-2 atom (δ_C 117.2 ppm) is correlated with H-3 atom (7.8 ppm), and the C-3 atom (δ_C 146.9 ppm) is correlated with H-2'/6' signals (δ_H 7.6 ppm). This results also prove that C-3 atom is directly attached to the benzene unit.

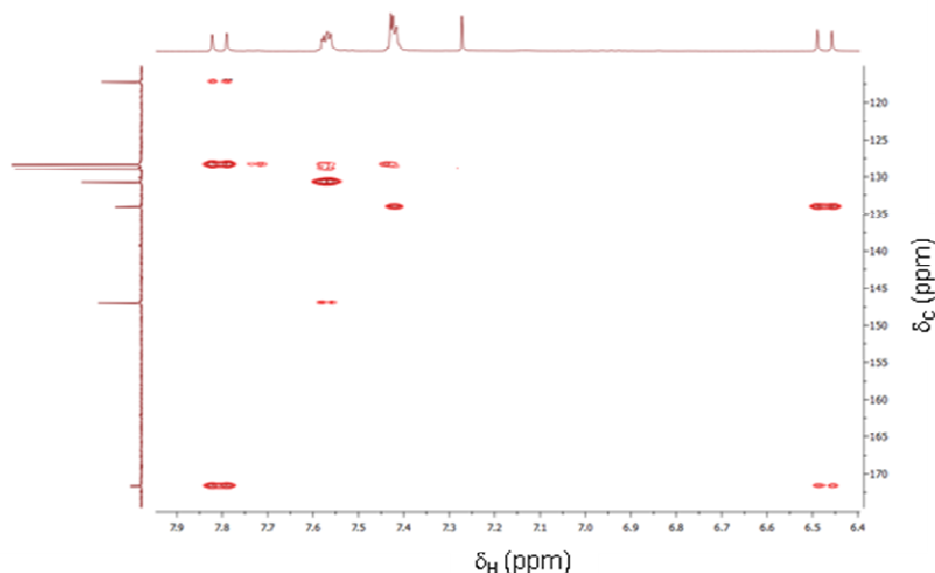


Figure 4 HMBC spectrum of H₇₁ fraction

Overall correlations observed in the HMBC spectrum are given in Table 4. An unpredicted correlation is still observed between C-2 atom and the *ortho* hydrogens. As shown in Fig. 1 and 2, fraction H₇₁ and H₆ still contain aliphatic impurities. An aromatic compound with similar structure as *trans*-cinnamic acid, but has C-2 atom of the aliphatic moieties directly attached to a monosubstituted benzene ring is predicted to be an impurity accounts for this correlation. However, from NMR analysis explained above, the chemical compound in fraction H₇₁ and H₆ can be predicted as crude *trans*-cinnamic acid, but still need further purification.

Table 4 Two (α) and three (β) bonds correlation between ^1H and ^{13}C NMR peak in the HMBC spectrum

^1H NMR	^{13}C NMR ppm	C-1 171.7	C-2 146.9	C-3 117.2	C-1' 134.0	C-2'/6' 128.3	C-3'/5' 128.9	C-4' 130.7
H-2	7.8	α	DB*	α		**		
H-3	6.4	β		DB	α			
H-2'/6'	7.6		β			DB	α	β
H-3'/4'/5'	7.4				β	α/β	DB	DB

*DB: directly bonded

**unpredicted correlation

trans-Cinnamic acid and *trans*-*p*-methoxycinnamic acid have been reported by Kuo *et al.* [10] in the ethanol extract of *D. repens* leaves grown in Taiwan. Ester of cinnamic acid with an iridoid glucoside moiety, namely durantoside I, has also been isolated from *D. erecta* leaves [17]. *trans*-Cinnamic acid itself is a common chemical compound found in plants.

Therefore, investigation on other chemical constituents in polar as well as nonpolar fractions of the methanol extract, which is characteristic of *D. repens* fruits in Indonesia, needs to be carried out in immediate study.

4. CONCLUSION

Phytochemical investigations on *D. repens* fruits' methanol extract from Jombang, East Java show the existence of tannin, saponin, alkaloid, flavonoid, and steroid. Crude *trans*-cinnamic acid has been isolated from acetone-soluble fraction of the extract as confirmed by 1D and 2D-NMR spectra analysis.

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