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Research Article

KAEMPFEROL FROM *STELECHOCARPUS BURAHOL*, (BL.) HOOK F. & TH. LEAVES AND XANTHINE OXIDASE INHIBITION ACTIVITY

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ABSTRACT

Objective: *Stelechocarpus burahol*, (Bl.) Hook f. & Th. is a plant widely distributed in Java Island of Indonesia. The aim of this study is to identify compounds from the leaves of *S. burahol* that exhibited activity as xanthine oxidase inhibitor (XOI).

Methods: The leaves were extracted with aqueous ethanol and hydrolyzed with HCl methanol, then, partitioned sequentially with chloroform and ethyl acetate. The ethyl acetate fractions were separated by column chromatography with cellulose as stationary phase and methanol 50% as mobile phase.

Results: Purification from this extracts afforded three compounds with one compound identified, namely kaempferol. The four compounds possessed as XOI with IC_{50} values ranging from 0.27 to 0.45 µg/ml.

Conclusion: Kaempferol exhibited the highest inhibition of 0.27 µg/ml.

Keywords: Kaempferol, Xanthine oxidase inhibitor, *Stelechocarpus burahol,* (Bl.) Hook f. & Th.

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INTRODUCTION

Stelechocarpus burahol, (Bl.) Hook f. & Th. is one of genus Annonaceae. *S. burahol* is tropical plant and is found in Indonesia, especially in Java Island [1]. *S. burahol* has been used traditionally as a contraceptive remedy in Javanese royal family. Its fruit contains toxic substances to fetus, that *S. burahol* pulp ethanol extract has potency for anti-implantation [2]. Fruit of *S. burahol* has been used as perfumery ingredient, to reduce uric acid levels in human body, prevent kidneys inflammation and abortifacient [3-5]. Susilowati (2000) [7] reported that *S. burahol* had potency as antihyperuricemia because the water extract of the leaves decreased plasma uric levels in rats, while Hening (2002) [8] mentioned that the water extract leaves of *S. burahol* decreased plasma uric levels in chickens.

The leaves of *S. burahol* contain flavonoid and polyphenols [8-10]. Based on the studies could be able to isolate aglycone flavonoid from the *S. burahol* leaves. Thus, it was relevant the antihyperuricemic activity of the aglycone flavonoid from this leaves by determine the inhibition activity of xanthine oxidase (XO) enzyme [11].

METHODS

Plant material, extraction, and isolation

The leaves of *S. burahol* were collected from Sleman District, Yogyakarta Province, Indonesia, in August 2013 and identified in the Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Gadjah Mada University. A voucher specimen was deposited in the Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Muhammadiyah University of Purwokerto. Leaves of *S. burahol* were dried and pulverized. It was extracted by maceration method using ethanol as a solvent (1:10) and shaken for 30 minutes, allowed stand for 24 hrs, then filtration. Continued extraction using the same solvent (1:4) and shaken for 30 minutes, let for 24 hrs, then filtrated. The filtrate collected was concentrated using rotary evaporator at a temperature 50-60°C. Ethanol extract (170 g) was hydrolyzed with 400 ml HCl 2 N: Methanol (1:1) by reflux method for 30 minutes, then added chloroform, shaken and allowed to stand 6 hrs. Water layer separated and followed by addition of ethyl acetate, shaken and allowed to stand for 6 hrs. The ethyl acetate fraction was collected and then separated using column chromatography method with cellulose as stationary phase and methanol 50% as mobile phase. The stationary phase used is 300 g using a column with a diameter of 6.5 cm manufacture of a column using a wet method, by mixing the stationary phase with a mobile phase with a ratio of 1:1 and then poured in a column chromatography, left overnight. The stationary phase was mixed with ethyl acetate fraction (28 g) with a ratio of 1:1, mixed homogeneously, placed on a stationary phase that has compacted in a column chromatography. Subfractions collected every 30 ml, 130 subfractions thus obtained. During the subfraction taking is monitored using a ultraviolet (UV) lamp in the column. There fluorescence of flavonoids in the column because the flavonoid compound fluoresces under UV light. After separation of active fractions by column chromatography, three compounds were obtained, and one was identified based on spectra data using ^{1}H- and ^{13}C-NMR.

XO inhibitory (XOI) activity assay

The inhibitory effect on XO was measured spectrophotometrically at 290 nm under aerobic condition, with some modifications, following the method reported by Ernawati and Susanti (2001) [12]. A well-known XOI, allopurinol (100 µg/ml) was used as a positive control for the inhibition test. XO activity determined by adding 200 mL of substrate (xanthine) 0.15 mM in a mixture of 100 mL of XO 100 mU/mL and 724 mL of phosphate buffer pH 7.5. XO activity determined by observing the rate of formation uric acid from xanthine by spectrophotometry at wavelength (λ) 290 nm from minute 0 up to 3 minutes at a temperature of 25°C. Data were obtained in the form of rate (Δ A290/minutes) [13].

The difference, at this stage the addition of 200 mL allopurinol at a concentration of 10 µg/mL to 100 µg/mL into a mixture of phosphate buffer, xanthine, and XO. In similar way, also determined the XOI activity by 200 mL of test solution (carried out using the orientation of concentration 10 µg/mL to 100 µg/mL).

RESULT AND DISCUSSION

The inhibitory effect of ethanolic extract of *S. burahol* leaves, chloroform fraction, ethyl acetate fraction, allopurinol, and isolates have been performed in Graphs 1 and 2.

Determination of the enzyme XOI activity using spectrophotometric method by observing the formation of uric acid at a wavelength of 290 nm. Xanthine has an absorption in the UV wavelength 260 nm (Nagao *et al*., 1999) [14] over 6 minutes uric acid formation reaction kinetics is linear (Van Hoorn *et al*., 2002) [15]. In this study, the rate of formation of uric acid linear 5 minutes, then at minute 6 start ramps. Thus, the determination of uric acid formation carried out during the first 4 minutes.

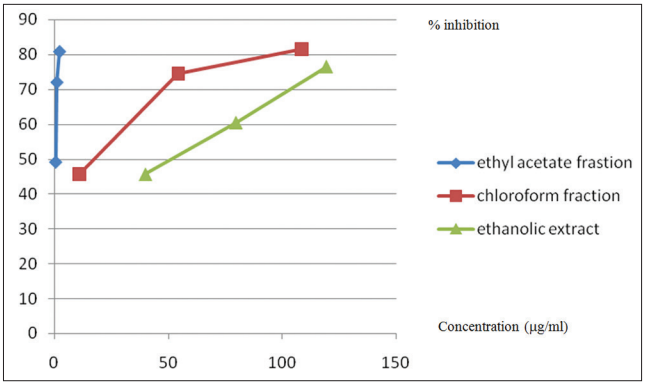
This study is based on a previous study conducted Purwantiningsih *et al*. (2010) [16] reported that the ethanol extract of Burahol leaves has antihiperurisemia activity *in vivo* in rats. The result of this research showed that ethanolic extract had IC_{50} 52.11 µg/mL. Ethyl acetate fraction had the higher activity with IC_{50} 0.31 µg/mL than chloroform fraction 9.78 µg/mL. Haddi and Marouf (2015) [17] reported ethyl acetate fraction from crude extract of *Pistacia lentiscus* L. (Family Anacardiaceae) leaves had higher XOI activity than butanolic, aqueous, and chloroformic fraction from the same crude extract. Ethyl acetate fraction had the lower activity IC_{50} 0.31 µg/mL than chloroform fraction 9.78 µg/mL (Fig. 1). Thus, ethyl acetate fraction was fractionated by column chromatography. The stationary phase used was cellulose and 50% methanol as mobile phase. Subfractions 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 and 130 test thin layer chromatography (TLC), using 3 times eluated acetic acid 30% as a mobile phase and cellulose as the stationary phase. Subfraction 50-79 was separated using preparative TLC to obtain three isolates (B1, B2, B3).

Further isolates tested activity as inhibitors of the enzyme XO, the results obtained (Table 1) that all isolates had activity to the enzyme XO, from the largest to the smallest activity were B1, B3, and B2 with

Table 1: IC_{50} of ethanolic extract of *S. burahol* leaves, chloroform fraction, ethyl acetate fraction, allopurinol, B1, B2, B3 isolates

Sample	IC_{50} (µg/mL)
Allopurinol	4.59
	4.29*
	3.16**
Ethyl acetate fraction	0.31
Chloroform fraction	9.78
Ethanolic extract	52.11
B1	0.27
B2	0.45
B3	0.30

*Septiningsih *et al*., 2012 [18], **Ernawati and Susanti, 2014 [12]



Graph 1: The xanthine oxidase inhibition effect of ethanolic extract of *Stelechocarpus burahol* leaves, chloroform fraction, ethyl acetate fraction

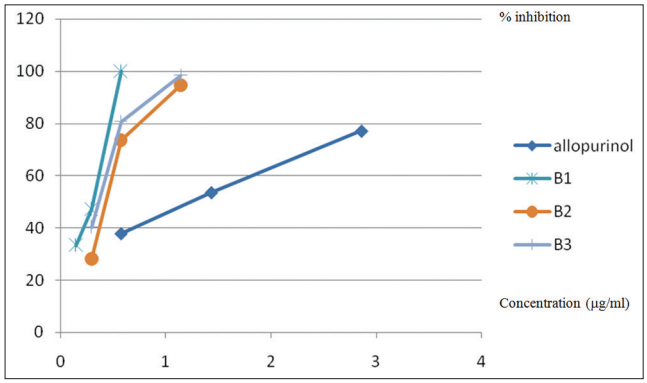
IC_{50} values of 0.27, 0.30, 0.45 µg/mL. Respectively, B1 isolates have IC_{50} similar to the ethyl acetate fraction (0.31 µg/mL), but lower than allopurinol (IC_{50} values 4.59 µg/mL) as positive control. Thus, the study continued to identify B1 isolates.

B1 isolates structure elucidation

The interpretation of the UV spectrum in methanol showed the absorption at a wavelength of 200-500 nm with a maximum wavelength of 366 nm and 265.5 nm, which the absorption flavonol kaempferol are 367 nm and 264 nm (Sikorka and Matlawska, 2001) [19], also appropriate according to Mabry (1970) [20] 250-280 nm and 350-385 nm for flavonol. The interpretation of the UV spectrum of B1 isolate was tabulated in Table 2.

The infrared spectrum B1 isolates showed broaded band in the region 3320/cm which indicates a hydroxyl group (–OH) (Supratman, 2010) [21], supported by the appearance of absorption at wave numbers 1090/cm was C-OH primer (Socrates, 1994) [22]. Strong absorption band formed sharply in the region 1663/cm indicates a group C=O (Stuart, 2004) [23]. Absorption on 1612/cm and 1569/cm was sharp and strong indicated C=C aromatic (Anderson and Markham, 2005) [24]. Absorption on 1441/cm and 834/cm which showed the bending on –CH (Mathias *et al*., 2000) [25]. Weak peak in the wave numbers 1008 and 1176/cm indicates a bond C-O (Millan, 1993) [26].

^{1}H-NMR (acetone-d6. 400 Hz) of the isolates B1 is used to identify the number and position of H contained in the molecular structure. In these data, there are several groups of signals consisting of 6 protons aromatic (Fig. 3). The emergence of doublet signals at 6.37 ppm (d. j=2 Hz) and 6.15 ppm (d. j=2 Hz). At position C-6 and C-8 show the position of the hydroxyl group at C-7 and C-5. The existence ortho coupling of two



Graph 2: The xanthine oxidase inhibition effect of allopurinol, B1, B2, B3 isolates

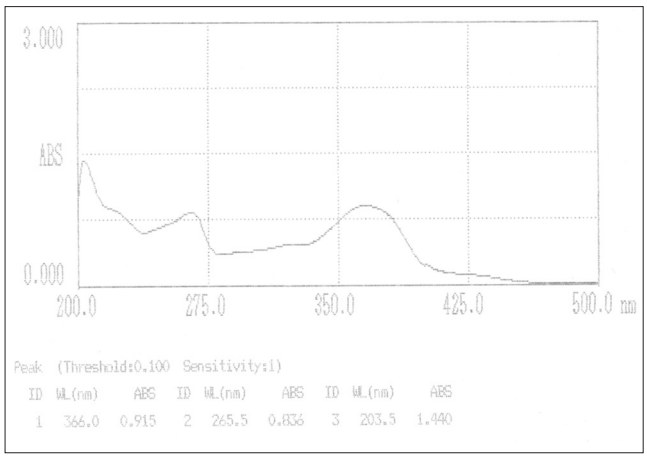


Fig. 1: The ultraviolet spectrum in methanol of B1 isolate

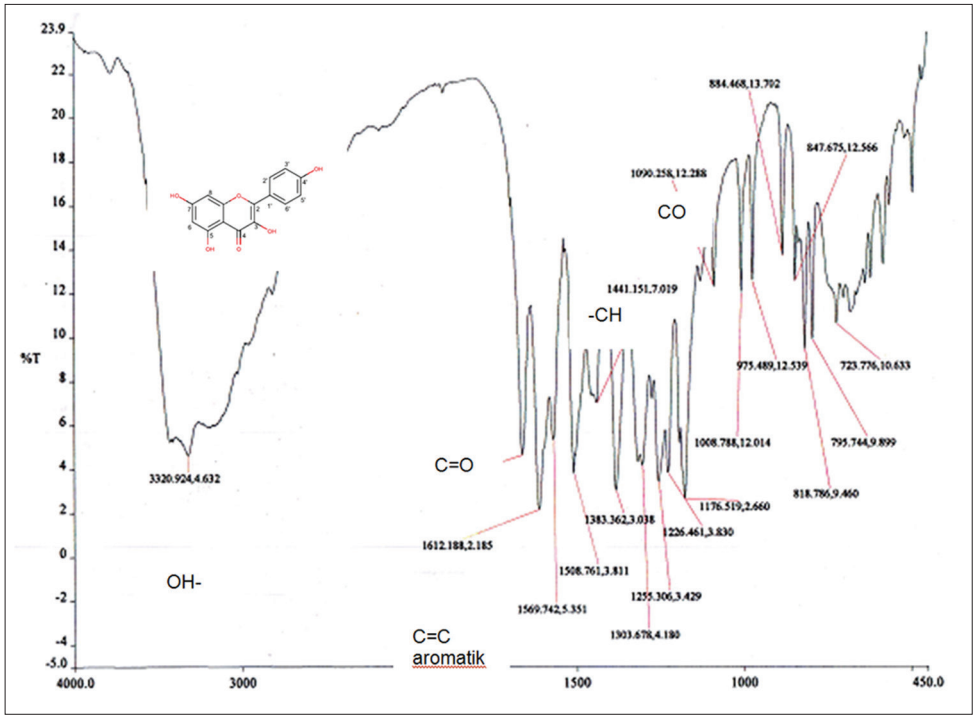


Fig. 2: Fourier transform infrared spectrum of B1 isolate in pellet KBr

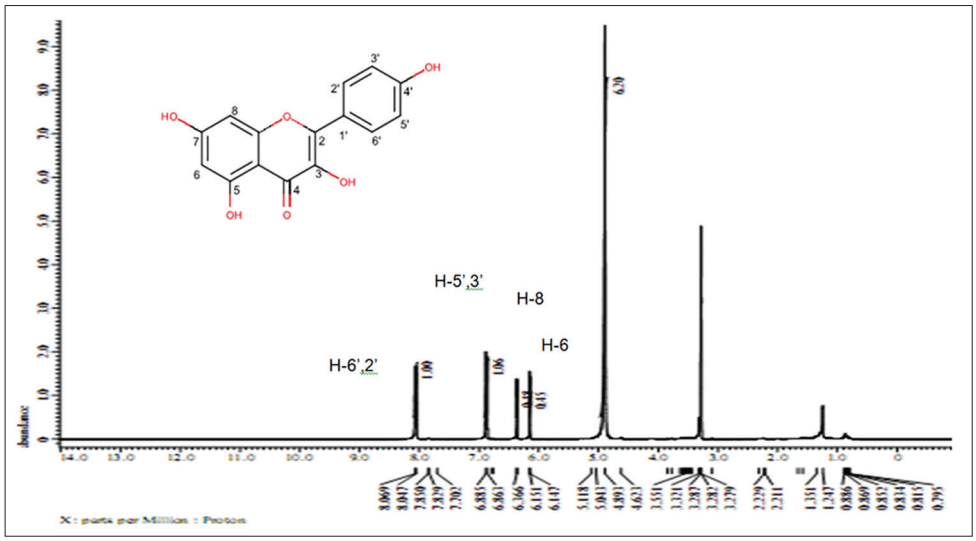


Fig. 3: ^{1}H-NMR spectrum of B1 isolate

Table 2: The UV spectra interpretation of B1 isolate

Sample	Isolate B1	Sikorska and Matlawska, 2001 [19]	Interpretation
In methanol +$AlCl_3$+$AlCl_3$/HCl	366 nm and 265,5 nm 424 nm and 269 nm 424 nm and 268 nm	367 nm and 264 nm 424 nm and 268 nm, 424 nm and 269 nm	Flavonol (Mabry, 1970) [20] 38 nm bathochromic shift (from 366 nm to 424 nm) on the addition of $AlCl_3$ showed an OH group at C number 5 The addition of hydrochloric acid does not cause a bathochromic shift it shows no ortho dihydroxy
+NaOAc+NaOAc/H_3BO_3	379.5 nm and 273 nm 365.5 nm and 266.5 nm	379.5 nm and 273 nm 365.5 nm and 266.5 nm	13.5 nm bathochromic shift (from 265.5 nm to 273 nm) on the addition of sodium acetate, showed the OH group at C number 7. The addition of boric acid does not cause a bathochromic shift it shows no ortho dihydroxy group in ring B

UV: Ultraviolet

doublet signals with the δ_H=8.07 ppm (d. j=9 Hz). 6.89 ppm (d. j=9 Hz) on position C-6'.2' and C-5'.3'.

^{13}C-NMR is used to indicate the number and position of C contained in the molecular structure showed (Fig. 4) there were 13 separate signals well to 15 carbon atoms chemical shifts at δ_C =93.09, 97.89, 103.19, 114.91, 122.37, 129.34, 135.81, 146, 66, 156.89, 159.20, 164.18, 164.24, 176.05 ppm all of which are carbon aromatic ring. There is a carbon of a carbonyl group –C=O on shift δ_C=176.05 ppm. There are two chemical shifts which abundant twice δ_C=114.91, 129.34 ppm.

Heteronuclear multiple-quantum correlation spectrum (Fig. 5) shows that the compound B1 shows that the proton –H6 aromatic chemical shifts d_H=6.15 ppm (s) bonded to carbon C6 δ_C=97.89 ppm. The proton –H8 aromatic chemical shifts δ_H=6.37 ppm (s) bonded to carbon C8 δ_C=93.09 ppm. The proton –H3',5' aromatic chemical shifts δ_H=6.89 ppm (d) bonded to carbon C3',5' δ_C=114.91 ppm. Then proton –H2',6' aromatic chemical shifts δ_H=8.07 ppm (d) bonded to carbon C2',6' δ_C=129.34 ppm.

Based heteronuclear multiple bond correlation (HMBC) spectrum (Fig. 6) shows that the compound B1 showed that aromatic carbon

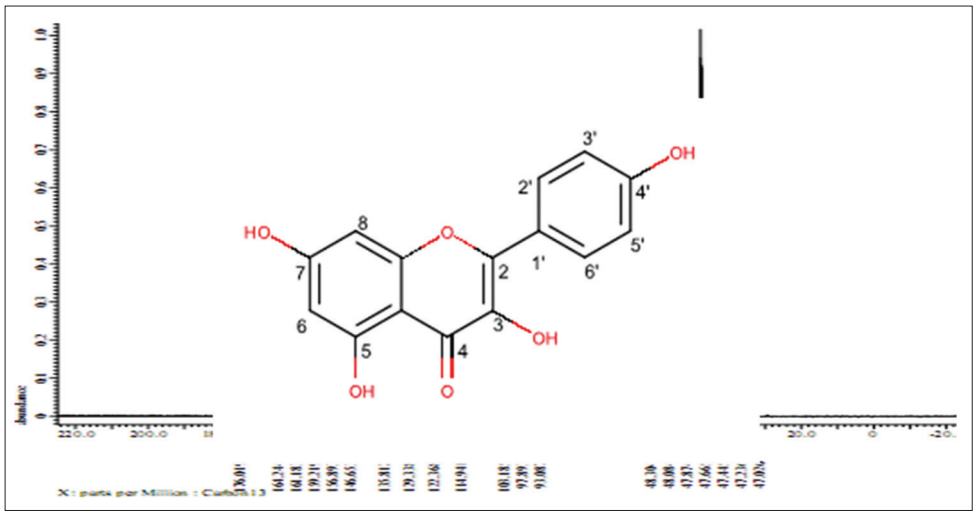


Fig. 4: ^{13}CNMR spectrum of B1 isolate

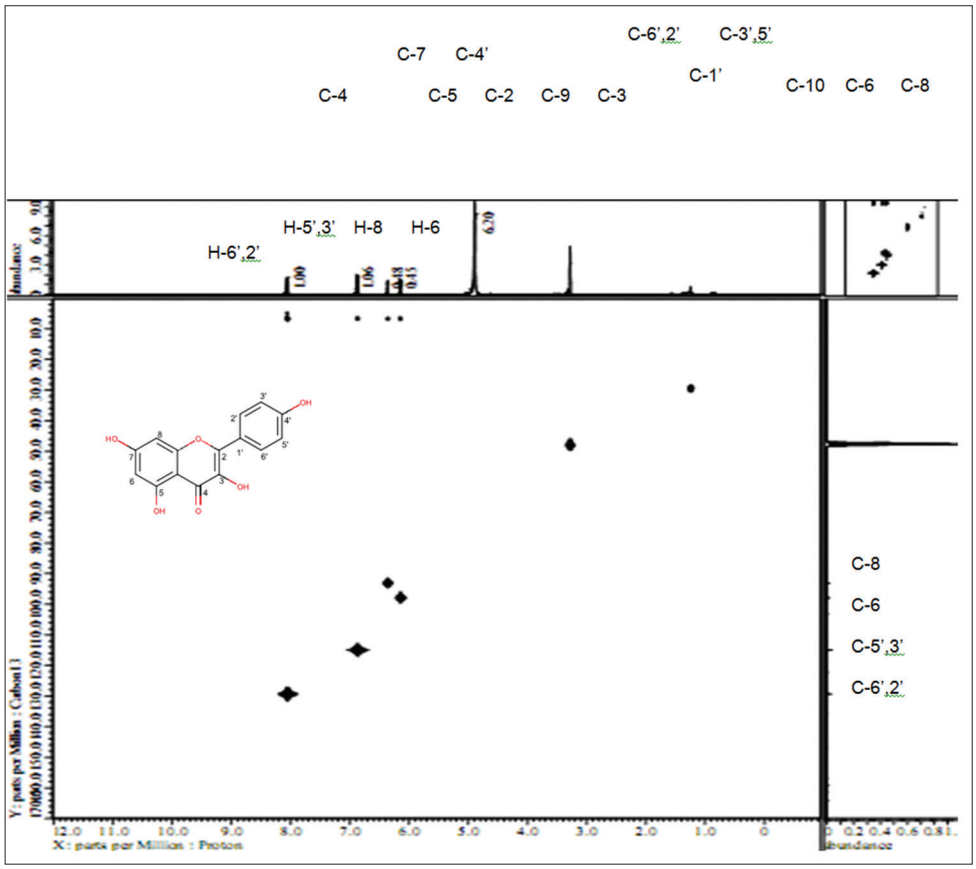


Fig. 5: Heteronuclear multiple-quantum correlation spectrum of B1 isolate

C4' at chemical shift δ_C=159.22 ppm interaction on proton H2',6',5',3' δ_H=8.07, 6.89 ppm. There carbon aromatic C2 at δ_C=156.89 ppm interaction on proton H2',6' δ_H=8.07 ppm. Aromatic carbon C2',6' at chemical shift δ_C=129.34 ppm interaction on proton H6',5',3' δ_H=8.07, 6.89 ppm. There carbon aromatic C1' at δ_C=122.37 interaction on proton H5',3' δ_H=6.89 ppm. Aromatic carbon C5',3' at chemical shift δ_C=114.91 interaction on proton H5',3',2',6' δ_H=8.07, 6.89 ppm.

Based HMBC spectrum (Fig. 7) shows that the compound B1 showed that aromatic carbon C7 at chemical shift δ_C=162.72 ppm interaction on proton H6,8 δ_H=6.15, 6.37 ppm. There carbon aromatic C5 at δ_C=164.18 ppm interaction on proton H8 δ_H=6.37 ppm. Aromatic carbon C9 at chemical shift δ_C=146.65 ppm interaction on proton H8 δ_H=6.37 ppm. There carbon aromatic C6 at δ_C=97.89 interaction on proton H8 δ_H=6.37 ppm. Aromatic carbon C8 at chemical shift δ_C=93.09 interaction on proton H6 δ_H=6.15 ppm.

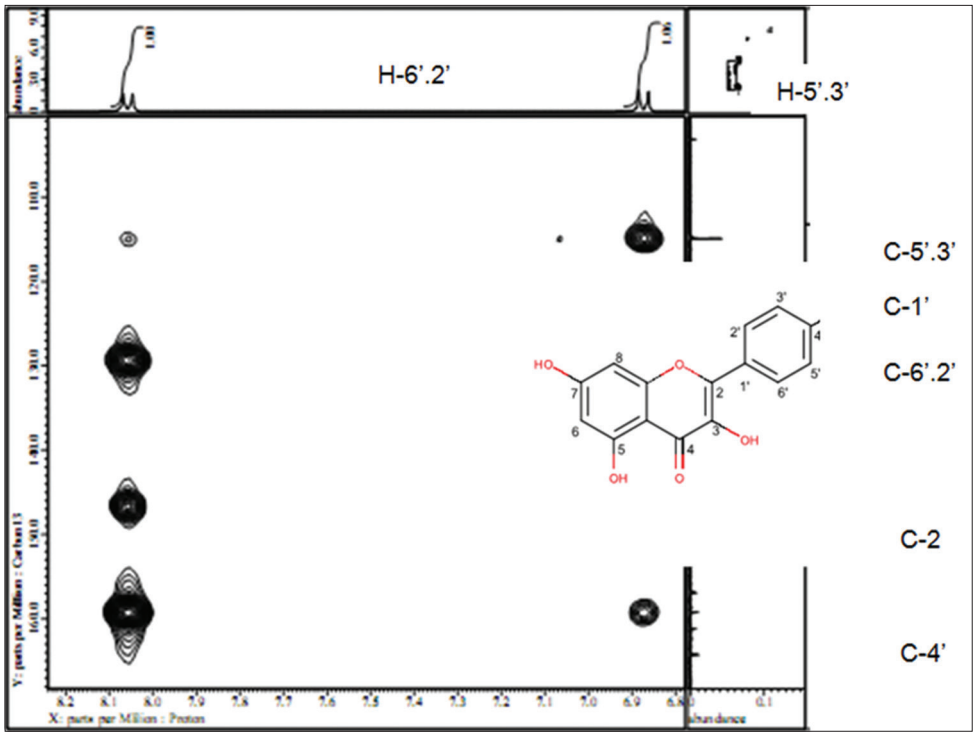


Fig. 6: Extended 1 heteronuclear multiple bond correlation of B1 isolate

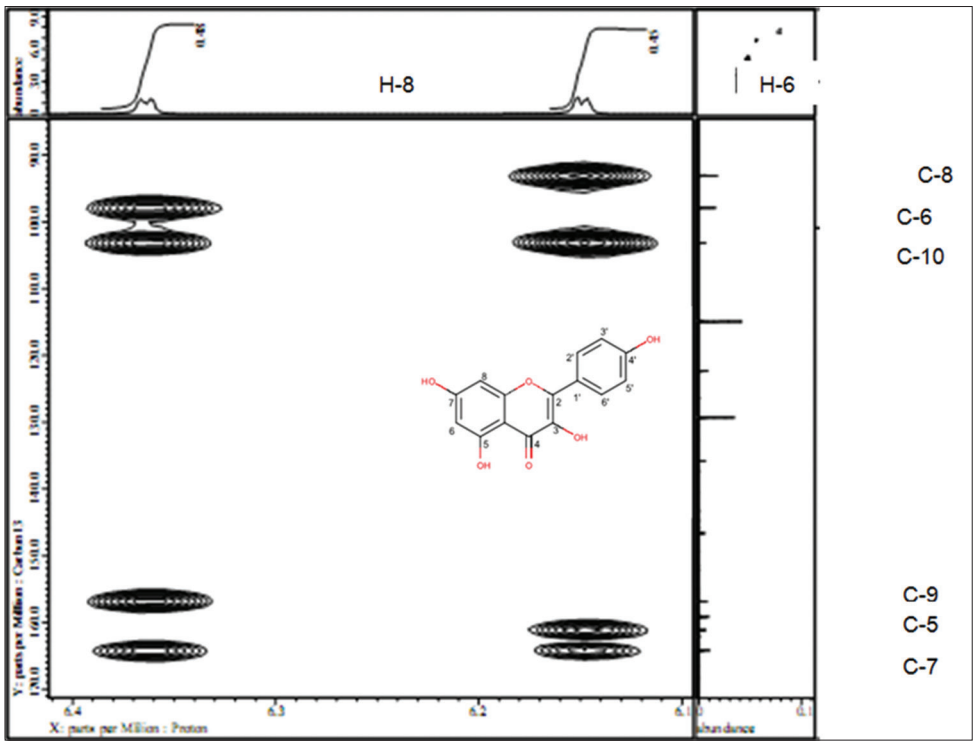


Fig. 7: Extended 2 heteronuclear multiple bond correlation of B1 isolate

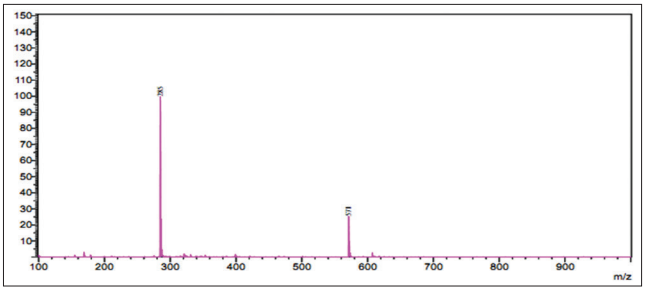


Fig. 8: MS m/z negative

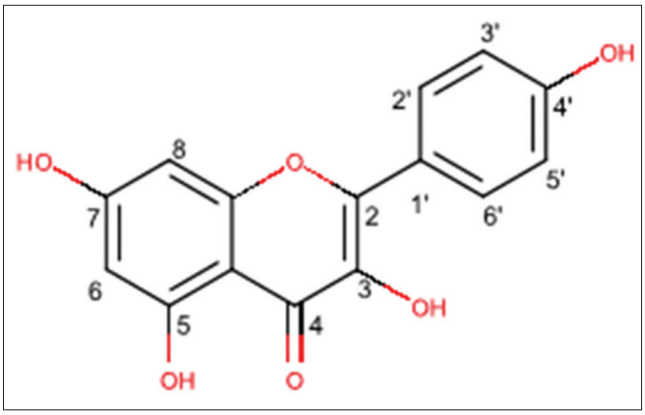


Fig. 9: Molecular structure of B1 compound

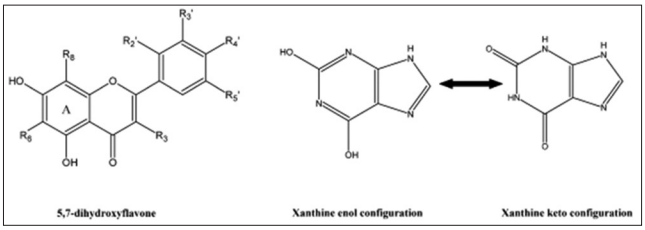


Fig. 10: Comparing the structural similarity enol form xanthine with 5.7-dihydroxylflavone. Structural similarity between enol form xanthine and 5,7- dihidroksiflavon show a similar binding site in the center of allosteric xanthine oxidase (Van Hoorn 2002)

Mass spectra of isolate B1 (Fig. 8) showed that m/z negative 285, molecular weight of kaempferol 286 g/mol. It is showed that isolate B1 is kaempferol.

Based on spectroscopic data analysis (Table 3), it can be concluded that the isolates B1 is kaempferol (Fig. 9) with molecular formula $C_{15}H_{10}O_6$.

According to Van Hoorn *et al.* (2002) [15], kaempferol structure on the part of the A ring is similar to the heterocyclic ring in the form of xanthine enol (Fig. 10). According to Lam *et al.* (2006) [27], allopurinol (positive control) has an activity of inhibiting xanthine as an allosteric inhibitor of XO by reducing the reactive groups on the XO (Massey *et al.*, 1970) [28].

According to Kumar *et al.* (2011) [29], inhibition of the enzyme XO from nonpurine compounds is lower than the side effects of purine-like compounds. Kaempferol is a nonpurine XOI, allopurinol is XO while purines.

CONCLUSION

In summary, the most active isolate which inhibits the XO (B1) with IC_{50} of 0.27 µg/ml is kaempferol. Moreover, three compounds isolated from the active fractions showing different XOI activities *in vitro* may contribute to antihyperuricemic effect. These result supported the antihyperuricemic activity of *S. burahol* leaves as health dietary candidate.

Table 3: Interpretation ^{13}C-NMR spectra of isolate B1

C	^{13}C chemical sift (ppm)		^{1}H chemical sift (ppm)		HMQC	HMBC
	Kaempferol*	Isolate B1	Kaempferol*	Isolate B1		
4	177.53	176.05				
7	162.72	164.24				H-6.8
5	160.74	164.18				H-6
4’	160.60	159.22				H-2’.6’.5’.3’
2	156.72	156.89				H-2’.6’
9	155.91	146.65				H-8
3	133.38	135.81				
2’	130.87	129.34	8.01	8.07	H-2’	H-6’.3’
6’	130.87	129.34	8.01	8.07	H-6’	H-2’.5’
1’	120.68	122.37				H-5’.3’
3’	115.04	114.91	6.86	6.89	H-3’	H-5’.2’
5’	115.04	114.91	6.86	6.89	H-5’	H-3’.6’
10	105.56	103.19				H-6.8
6	99.66	97.89	6.20	6.15	H-6	H-8
8	94.258	93.09	6.25	6.37	H-8	H-6

*Sikorska and Matlawska. 2001, [17]. HMQC: Heteronuclear multiple-quantum correlation, HMBC: Heteronuclear multiple bond correlation

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