Structural Elucidation of Two Flavonoid Compounds and Screening On Antipyretic Activity from the Rhizome of *Zingiber zerumbet* (L.) Roscoe ex Sm.

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Abstract

Zingiber zerumbet (L.) Roscoe ex Sm..is a long history of important traditional medicinal herbs for antipyretic, anti-inflammatory, anti-tumor, antioxidant and antimicrobial activities. In this present study was designed to evaluate the antipyretic activity of ethanolic extract by Brewer's yeast induced pyrexia method, isolation of two flavonoid compounds (3″, 4″-*O*-diacetylafzelin and 2″, 4″-*O*-diacetylafzelin) by column chromatographic method and identification of these two flavonoid compounds by using modern spectroscopic methods ^{2D} NMR (^{13}C NMR, ^{1}H NMR, HMBC, HSQC, ^{1}H-^{1}H COSY and EI MS). Ethanolic extract by changing concentration and zerumbone compounds can be reduced fever nearly standard drug paracetamol.

Keywords:*Zingiber zerumbet* (L.) Roscoe ex Sm. 3″,4″-*O*-diacetylafzelin, 2″, 4″-*O*-diacetylafzelin, antipyretic activity

Introduction

Zingiber zerumbet (L.) Roscoe ex Sm.is a smooth, erect and herbaceous plant. Root stocks are large and tuberous, pale yellow with leafy stem is 0.6 to 2 m high. Phytochemical investigations on this plant have revealed the isolation of several sesquiterpenes, flavonoids and aromatic compounds, phenolic compounds, tannins, amino acids, alkaloids and so on. Isolated bioactive compounds: humulene, monoterpenes and zerumbone can be extracted from the essential oil. (Z)-nerolidol, phenolic compounds, saponins and terpenoids have been isolated from extracts of rhizomes, stems, leaves, and flowers (Jiang, 2013). The family *Zingiberaceae* are widely cultivated throughout the tropics including Southeast Asia, Korea, India and Bangladesh for its medicinal properties. It is used in traditional medicine as a cure for swelling, loss of appetite, lumbago, diabetes, inflammation, chest pain, rheumatic pains, bronchitis, dyspepsia and sore throat. The juice of the boiled rhizomes has also been used in indigenous medicine for worm infestation in children (Kader, 2011).

Materials and Methods

General experimental procedures

The UV spectra of isolated compounds in methanol were recorded with a Shimadzu UV-240, UV-visible spectrophotometer (Japan). The FT IR spectra of all isolated compounds were taken with KBr pellets and recorded on Shimadzu FT IR 8400 Fourier Transform Infrared Spectrometer. The ^{1}H NMR spectra of two isolated compounds were determined in $CD_3OD$and acetone using TMS as an internal standard and recorded on INOVA-600 (600 MHz for proton). The ^{13}C NMR spectra of isolated compounds were determined in CD_3OD using TMS as internal standard and recorded on INOVA 500 (125 MHz for carbon). COSY, HMBC and HSQC spectra of isolated compounds were determined CD_3OD recorded on

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INOVA 600 (600 MHz for proton and 125 MHz for carbon). The antipyretic activity of extracts was evaluated using Brewer's yeast induced pyrexia in albino rats.

Plant materials

Zingiber zerumbet (L.) Roscoe ex Sm. was collected from the buffer zone in Hlawga Park, Yangon Division. The sample was authenticated by botanist of Department of Botany, University of Yangon.

Family : *Zingiberaceae*
Botanical name : *Zingiber zerumbet* (L.) Roscoe ex Sm.
English name : Wild Ginger, Shampoo Ginger
Myanmar name : Linn-nay-gyi
Local name : Gank-eik

Figure 1.Photographs of the rhizome of *Zingiber zerumbet* (L.) Roscoe ex Sm.

Procedure for Extraction and Isolation of Two Flavonoid Compounds

The air-dried powder samples rhizome of *Z. zerumbet* (L.) Roscoe ex Sm. (100 g) was macerated with ethyl acetate (3 x 1L) at room temperature for about two weeks by the percolation method. The ethyl acetate extract (9 g) of *Z. zerumbet* (L.) Roscoe ex Sm. was chromatographed over silica gel as stationary phase using Pet-ether: EtOAc (19:1) as mobile phase to afford 7 pooled fractions. From Pet-ether: EtOAc (1:4) fraction, flavonoid rich fraction was obtained as yellowish amorphous powdered. And then, these flavonoid rich fractions were subjected to column chromatography over silica gel (70-230 mesh) eluted with Pet-ether and EtOAc of increasing polarity ratio of (2:1, 1:1, 1:2, 1:4, and finally with MeOH). From fraction Pet-ether: EtOAc (1:1), two yellow amphorous flavonoid compounds were obtained. TLC chromatograms were visualized under UV-lamp (254-365 nm) as well as by spraying with 5 % $FeCl_3$ reagent and give yellow colour for a mixture of two flavonoid compounds (compound I and compound II).

Procedure for Antipyretic Activity

Animals of either sex were divided into seven groups containing in each group for this experiment. The normal body temperature of each rat was measured rectally at one hour interval on a thermometer probe and recorded. The antipyretic activity of extract was evaluated using Brewer's yeast induced pyrexia in albino rats. Before yeast injection, the basal rectal temperature of rats was recorded and after recording animals was given subcutaneous injection of 10 mL/kg of 15% w/v yeast suspended in 0.5% w/v methyl cellulose solution for elevation of body temperature of rats. Rats were then returned to their housing cages. At the 19 h after

yeast injection, standard drug and test drugs were administered into different groups. Distilled water at dose of 10 mL/kg was administered orally to the control groups of animals and paracetamol at dose of 500 mg/kg was administered orally to standard group of animals. 98% ethanolic extract of *Z. zerumbet* (L.) Roscoe ex Sm. was administered orally at doses of 1 g/kg, 2 g/kg, 4 g/kg and zerumbone compound (MohMohMyint, 2012) was administered orally at a dose of 0.75 g/kg of body weight to five groups respectively. Rectal temperature was recorded by the thermometer probe at 0, 1, 2, 3, 4, 5 h after drug administration (Somchit, 2003).

Results and Discussion

Identification of Compound I and Compound II

A mixture of compound I and II were obtained as yellow amorphous powdered from ethyl acetate extract by column chromatography on a silica gel column eluted successively with pet-ether and ethyl acetate. It gave a yellowish spot on TLC with 5% $FeCl_3$ reagent. The $R_f$value was 0.5 with chloroform: methanol (9:1). Therefore, these compounds may be flavonoids.

In the UV spectrumin MeOH, a mixture of compound I and II showed at 264, 292 (sh) and 341 nm due to the absorption bands matched with that of 3-OH substituted flavonoids of compound I and compound II (Markham, 1982).

The ^{1}HNMR spectrumin CD_3OD showed resonances for two singlet aromatic protons [δ_H 6.20 (1H, *d*, H-6), 6.40 (1H, *d*, H-8)], four *ortho*-coupled aromatic protons [δ_H7.79 (2H, *d*, *J*= 8.5 Hz, H-2′, 6′), 6.98 (2H, *d*, *J*= 8.5 Hz, H-3′, 5′)]. These proton NMR data are in good agreement with literature value of kaempferolaglycone (Ozden, 1998). The presence of anomeric protons was appeared as a doublet at δ 5.63 and 5.56 ppm were suggesting two sugars units. These anomeric protons had a coupling constant of 2 Hz, confirming the α-orientation of sugar moiety. These sugars were α-L-rhamanoses (δ 99.9 and 101.8) suggested by the distinct anomeric protons at δ5.60 (*J*= 2 Hz) and δ 5.52 (*J*= 2 Hz) and methyl protons at δ 0.80 ppm.

In ^{1}HNMR spectrain acetone, the mixture of compound I and II displayed a downfield resonance at δ_H 12.6 attributed to a chelated hydroxyl proton. In the aromatic region, two doublets of doublets at δ 7.90 and 7.04 ppm(each 4-H, *J*=4, 9 Hz) andtwo meta-coupled doublet at δ6.30 and 6.50 (each 2-H, *J*=1, 2 Hz) indicated the presence of two sets of flavonoid aglycone. The doublets of δ5.63 and 5.56ppm were shown for the presence of two anomeric protons in these compounds. There are four singlet protons at δ2.05, 2.01, 1.97 and 1.95ppm integrating for four acetate methyl groups in sugar moiety.

In the ^{13}C NMR spectrum of isolated compound I (CD_3OD, 125 MHz), a total of 25 carbons were observed in this spectrum. Among these, 15 carbons were observed for flavone in this spectrum. Carbonyl carbon peaks was found at δ 179.4 ppm. Eight quaternary carbon peaks at δ 166.0, 158.6, 163.2, 161.7, 116.7, 122.1 and 105.8 ppm corresponded to C-7, C-9, C-5, C-4′, C-3′, C-1′ and C-10. In addition the peaks represent six methine carbons were observed at δ 131.9, 116.7, 131.9, 135.1, 100.6 and 94.9 ppm which were corresponded to C-6′, C-5′, C-2′, C-3, C-6 and C-8. The remaining sugar carbons appearing at δ17.6–73.0 were characterized by NMR data and showed the sugars to be pyranose form for compound I (Agrawal, 1989).

In the ^{13}C NMR spectrum of isolated compound II(CD_3OD, 125 MHz),a total of 25 carbons were observed in this spectrum. Among these, 15 carbons were observed for flavone in this spectrum.Carbonyl carbon peak was found at δ 179.2 ppm. Eight quaternary carbon peaks at δ 166.0, 158.6, 163.2, 161.8, 116.7, 122.36 and 105.9 ppm corresponded to C-7, C-9, C-5, C-4′, C-3′, C-1′ and C-10. In addition the peaks represent six methane carbons that were observed at δ 131.9, 116.7, 131.9, 135.0, 100.0 and 94.9 ppm which were corresponded to C-6′, C-5′, C-2′, C-3, C-6 and C-8. In sugar moiety, C-2″, C-3″, C-2″, C-4″ and C-5″ appeared at δ 69.6, 73.0, 71.8 and 68.9 ppm, respectively. The two anomeric carbon signals were observed at δ99.9 and 101.8 ppm for compound I and II.

In the HSQC spectrum, the four methyl protons signals at δ 2.09, 2.10, 2.03 and 1.98 ppm were correlated with the carbons at δ172.8, 172.2, 172.2 and 171.8 ppm. So, these compound I and II are very similar compounds and each has two methyl acetate groups. The aromatic protons at δ 6.20 and 6.40ppm were connected with the carbons at δ100.6 and 94.5ppm pointing out the presence of aromatic A ring for compound I.The aromatic protons at δ 6.20 and 6.40 ppm were joined with the carbons at δ100.0 and 94.9ppm pointing out the presence of aromatic A ring for compound II.

In the HMBC spectrum of compound I and compound II, significant long range correlation in between H-6/C-5, C-7, C-8, C-10; H-8/C-6, C-7, C-9, C-10; H-2′, H-6′/ C-4′, C-2 and confirmed the presence of kampferolaglycone and significant long range correlations were observed between H-1″/C-3 and C-2″; H-5″/C-4″, C-3″, C-6″ indicated the presence of sugar moiety for compound I and compound II. The presence of L- rhamnosyl moiety was further supported by the presence of two secondary methyl groups at δ0.88 ppm (*J*=3 Hz) as well as the absence of oxymethylene groups at C-6″ position of the sugar unit. In compound I, the significant long range correlation between H-3″/ carbonyl carbon at δ 171.8 ppm and H-4″/ carbonyl carbon at δ 172.2 ppm showed the presence of two acetyl groups at C-3″ and C-4″ of sugar moiety. In compound II, the significant long range correlation between H-2"/ sugar acetate carbonyl carbon at δ 172.2 and H-4″/ sugar acetate carbonyl carbon at δ 171.8 ppm indicated the presence of two acetyl groups at C-2″ and C-4″ of sugar moiety.

The aromatic protons at δ7.79 ppm (*dd*, *J*=4, 9 Hz) and 6.98 (*dd* , *J*= 4, 9 Hz) each being integrated for two protons, indicated the presence of a symmetrical substitution in ring B correlated with C-6′(δ 131.9 ppm) and C-5′ (δ 116.7 ppm) for compound I and II. The C-8 (δ 94.9) correlated with H-6 (δ 6.20) and C-6 (δ 100.6 and δ 100.0) correlated with H-8 (δ 6.40) for compound I and II.

In the COSY spectrum for compound I and compound II, the correlation of H-6 and H-8; H-2″ and H-3″; H-3″ and H-4″; H-4″ and H-5″; H-3′ and H-5′ and H-5″ and H-6″ can be seen.

In the EI MS spectrum of isolated compound I and II, the molecular ion peak [M^+] was observed at m/z=286 indicating the compound has molecular weight 286 and which was constantly with molecular formula $C_{15}H_{10}O_6$. The long base peak 43 was shown acetate group in these compounds. The peak 231.1 was attributed to sugar acetate. So, these flavonoid compounds may be attached to one sugar group on each one (Jang,*et al*., 2004).

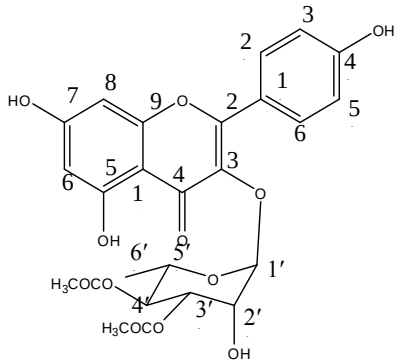
According to the literature values and observed values, compound I was 3″,4″-*O*-diacetylafzelin and compound II was 2″,4″-*O*-diacetylafzelin.

Compound I (3″, 4″-*O*-diacetylafzelin)

Yellow amorphous, 3″, 4″-*O*-diacetylafzelin, R_f= 0.5, UV(MeOH) λ_{max}/nm: 264, 292 (sh), 341; FT IR(KBr) υ_{max}/cm^{-1}: 3500, 3080, 2900, 1732, 1654, 1604, 1504, 1442, 1356, 1215, 1049, 837; ^{1}H NMR (600 MHz,CD_3OD): δ= 6.20 (1H, *d*, *J*= 1 Hz, H-6), 6.40 (1H, *d*, *J*= 2 Hz, H-8), 7.79 (1H,*dd*, *J* = 4, 9 Hz, H-2′ and H-6′), 6.98 (1H, *dd*, *J*= 4,9 Hz, H-3′and 5′), 5.60 (1H, *d*, *J* = 2Hz, H-1″), 4.35 (1H, broad *s*, H-2″), 5.16 (1-H, *dd*, *J*= 3,10 Hz, H-3″), 2.09 (3-H, *s*, H-3″-CH_3), 5.00 (1-H, *t*, *J*= 10 Hz, H-4″), 2.10 (3-H, *s*, H-4″-CH_3), 3.28(1-H, *m*, H-5″), 0.80 (3-H, *s*, H-6″CH_3).^{1}H NMR (600 MHz, acetone): δ= 6.03 (1H, *d*, *J*=1 Hz, H-6), 6.50 (1H, *d*, J = 2Hz, H-8), 7.90 (1H,*dd*, J = 4, 9 Hz, H-2′ and H-6′), 7.04 (1H, *dd*, *J*= 4,9 Hz, H-3′and 5′), 12.6 (1H, *s*, H-5′), 5.63 (1H, *d*, *J* = 2Hz, H-1″), 4.41 (1H, *broad s*, H-2″), 5.17 (1-H, *dd*, *J*= 3,10 Hz, H-3″), 2.05 (3H, *s*, H-3″-CH_3), 5.06 (1-H, *t*, J = 10 Hz, H-4″), 2.01 (3-H, *s*, H-4″-CH_3), 3.38 (1-H, *m*, H-5″), 0.81 (3-H, *s*, H-6″CH_3).^{13}CNMR (125 MHz, CD_3OD): δ=159.4 (C-2), 135.1 (C-3), 179.4 (C-4), 163.2 (C-5), 100.6 (C-6), 166.0 (C-7), 94.9 (C-8), 158.6 (C-9), 105.8 (C-10), 122.1 (C-1′), 131.9 (C-2′), 116.7 (C-3′), 161.7 (C-4′), 116.7 (C-5′), 131.9 (C-6′), 101.8 (C-1″), 69.6 (C-2″), 73.0 (C-3″), 171.8 (C-3″acetate), 20.8 (C-3″methyl), 71.8(C-4″), 172.2 (C-3″) 20.8 (C-3″methyl), 69.8(C-5″), 17.6(C-6″). The HMBC spectrum of 3″, 4″-O-diacetylafzelin showed the following key correlations: C-2/H-6, C-5/H-6, C-6/H-8, C-8/H-6, C-2′/H-3′& H-5′, C-3′/H-2′& H-6′, C-6′/H-2′, C-3″acetate/H-4″.

Compound II (2″, 4″-*O*-diacetylafzelin)

Yellow amorphous, 2″, 4″-*O*-diacetylafzelin, R_f= 0.5, UV (MeOH), λ_{max}/nm: 264, 292 (sh), 341; FT IR(KBr) υ_{max}/cm^{-1}: 3500, 3080, 2900, 1732, 1654, 1604, 1504, 1442, 1356, 1215, 1049, 837; ^{1}H NMR (600 MHz, CD_3OD): δ= 6.20 (1H, *d*, *J*= 1Hz, H-6), 6.40 (1H, *d*, J = 2Hz, H-8), 7.79 (1H,*dd*, J = 4, 9Hz, H-2′ and H-6′), 6.98 (1H, *dd*, *J*= 4,9 Hz, H-3′and 5′), 5.52 (1H, *d, J*=2Hz, H-1″), 5.42 (1H, broad *s*, H-2″), 4.02 (1-H, broad *s*, *J*= 3,10 Hz, H-3″), 2.03 (3-H, *s*, H-2″-CH_3), 4.78 (1-H, *t*, J = 10Hz, H-4″), 1.98 (3H, *s*, H-4″-CH_3), 3.28 (1-H, *m*, H-5″), 0.80 (3-H, *s*, H-6″CH_3).^{1}H NMR (600 MHz, acetone): δ= 6.30 (1H, *d*, *J*= 1Hz, H-6), 6.50 (1H, *d*, J=2 Hz, H-8), 7.90 (1H,*dd*, J = 4, 9Hz, H-2′ and H-6′), 7.04 (1H, *dd*, *J*= 4,9 Hz, H-3′and 5′), 12.6 (1H, *s*, H-5′), 5.56 (1H, *d, J* = 2Hz, H-1″), 5.47 (1H, *dd*, J = 1.5, 3Hz, H-2″), 4.28 (1-H, *dd*, *J*= 3,10 Hz, H-3″), 2.03 (3-H, *s*, H-2″-CH_3), 4.76 (1-H, *t*, *J* = 10Hz, H-4″), 1.95 (3-H, *s*, H-4″-CH_3), 3.48 (1-H, *m*, H-5″), 0.81 (3H, *s*, H-6″CH_3).^{13}CNMR (125MHz, CD_3OD): δ=159.4 (C-2), 135.0 (C-3), 179.2 (C-4), 163.2 (C-5), 100.0 (C-6), 166.0 (C-7), 94.9 (C-8), 158.6 (C-9), 105.9 (C-10), 122.4 (C-1′),131.9 (C-2′), 116.7 (C-3′), 161.8 (C-4′), 116.7 (C-5′), 131.9 (C-6′), 99.9 (C-1″), 72.9 (C-2″), 69.6 (C-3″), 172.2 (C-3″acetate), 20.7 (C-3″methyl), 74.7 (C-4″), 171.8 (C-3″), 20.6 (C-3″methyl), 69.4 (C-5″), 17.6 (C-6″) The HMBC spectrum of 2″, 4″-*O*-diacetylafzelin showed the following key correlations: C-2/H-6, C-5/H-6, C-6/H-8, C-8/H-6, C-2′/H-3′& H-5′, C-3′/H-2′& H-6′, C-6′/H-2′, C-3″acetate/H-4″.



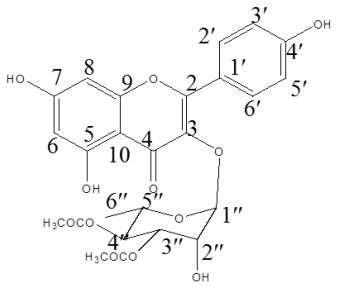


Figure 2.Structures of3″, 4″-*O*-diacetylafzelin and 2″, 4″-*O*-diacetylafzelin

Investigation of Antipyretic Activity

The antipyretic activity of 98% ethanolic extracts and zerumbone of the *Z. zerumbet* (L.) Roscoe ex Sm. were tested on yeast induced pyrexia rats.Treatment with ethanolic extracts at the doses of 4 g/ kg, 2 g/kg and 1 g/kg body weight; zerumbone at dose of 750 mg/kg and paracetamol at dose of 500 mg/kg decreased body temperature of yeast induced rats. The reduction in rectal temperature of pyrexia rats, treated with different doses of 98% ethanolic extracts and zerumbone of the drugs, were recorded at 20 h, 21 h, 22 h, 23 h and 24 h post administration. The reduction in rectal temperature of treated animals at each interval was compared with that of untreated pyrexia rats. The results obtained from both standards and extracts treated groups were compared with the control group. After administration one hour, a significant reduction in the yeast elevated rectal temperature was observed in the ethanolic extracts of *Z.zerumbet* ($p < 0.005$) at the dose of 1g/kg, ($p < 0.05$) at the dose of 2 g/kg,($p < 0.001$) at the dose of 4 g/kg and ($p < 0.005$) at the dose 750 mg/kg zerumbone.

Table 1. Effect of the Ethanolic Extract and Zerumbone of *Zingiber zerumbet* (L.) Smith. and Paracetamol on Brewer's Yeast Induced Fever in Rats

Extract	**Dose**	**Body temperature (mean ±SEM in °C)**					
		0 h	**19 h**	**20 h**	**21 h**	**22 h**	**23 h**
Control (n=6)	10 mL/kg	36.92 ± 0.15	37.67 ± 0.11	37.67 ± 0.17	37.5 ± 0.13	37.08 ± 0.15	36.83 ± 0.11
Paracetamol (n=6)	0.5 g/kg	36.83 ± 0.11	37.58 ± 0.12	35.92 ± 0.15$^{\#}$	35.37 ± 0.29$^{\#}$	34.87 ± 0.19$^{\#}$	35.2 ± 0.31$^{\#}$
Z. zerumbet (L.) Smith. (n=6)	1 g/kg	36.8 ±0.11	37.25 ± 0.11*	36.33 ± 0.31***	35.92 ± 0.44***	36.08 ± 0.35**	36.08 ± 0.30*
	2 g/kg	36.92 ± 0.14	37.65 ± 0.13	36.47 ± 0.43*	36.3 ± 0.31**	36.62 ± 0.12	36.73 ± 0.12**
	4 g/kg	36.98 ± 0.1	37.78 ± 0.1	36.68 ± 0.12$^{\$}$	36.37 ± 0.24**	36.32 ± 0.37	36.55 ± 0.38
Zerumbone (n=6)	0.75 g/kg	36.87 ± 0.08	37.62 ± 0.1	36.87 ± 0.15***	36.57 ±0 .22***	36.33 ± 0.21*	36.45 ± 0.32

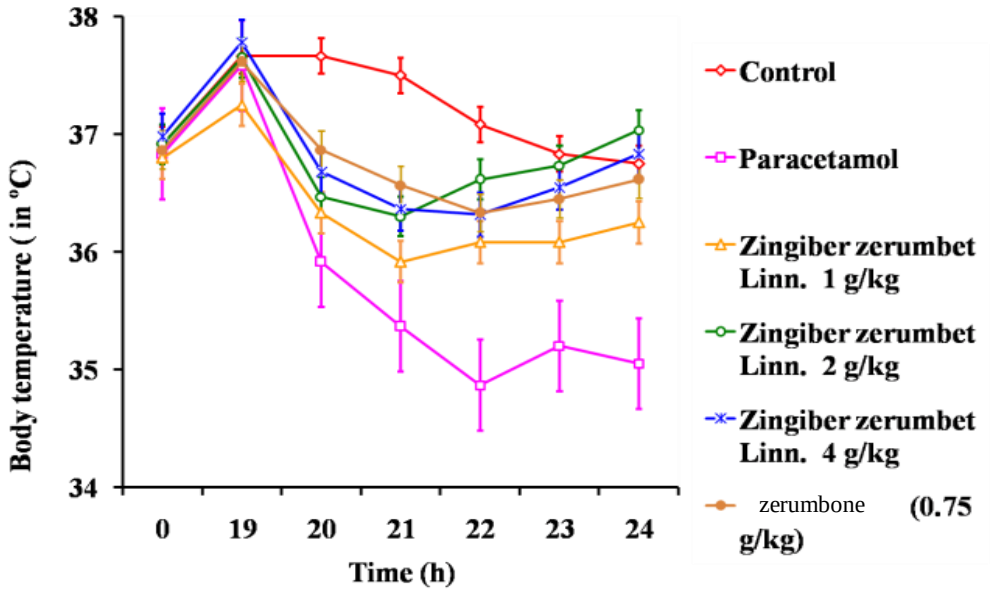


Figure 3Comparison of the effects on body temperature between test drugs plants extracts of *Z. zerumbet* and paracetamol

Conclusion

In the chemical investigation, 3 ″, 4″-*O*-diacetylafzelin (compound I) and 2″, 4″-*O*-diacetylafzelin (compound II) were isolated from the ethyl acetate extract of *Z.zerumbet* (L.) Roscoe ex Sm. These isolated compounds were separated by using column chromatographic method. They were identified by using of their R_f values and modern spectroscopic techniques such as UV-vis, FT IR, ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC and EI MS.

The antipyretic activity of 98% ethanolic extracts of rhizome sample and pure compound zerumbone isolated from *Z. zerumbet* were screened in adult albino rats by using yeast-induced pyrexia method. The reduction in rectal temperature of treated animals at each interval was compared with that of untreated groups and standard drug paracetamol. Zerumbone and ethanolic extract showed the active in antipyretic activity than the other concentration.

According to these findings, present study indicates that the two isolated flavonoid compounds can be identified as 3 ″, 4″-*O*-diacetylafzelin and 2″, 4″-*O*-diacetylafzelin. The extracts and isolated compounds of *Z. zerumbet*could are applied in the treatment of fever.

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