



Assessment of biological activity *in vitro* and phytochemical constituents from *Rosa hybrida* leaves

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Abstract The health benefits of roses have been demonstrated previously. This study aimed to isolate phytochemical constituents from the leaves of *Rosa hybrida* (LRH) and identify their chemical structures by ¹H-NMR, ¹³C-NMR, and mass spectrometry. Fractionation was carried out using open-column chromatography to isolate the compounds in *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. Four phytochemical constituents from LRH were isolated and identified, including oleanolic acid, uncariic acid, quercitrin (quercetin 3-*O*-rhamnoside), and afzelin (kaempferol 3-*O*-rhamnoside). Furthermore, the study explored the antioxidant properties of the extract and assessed the total contents of polyphenols and flavonoids. The LRH extract exhibited strong antioxidant activity, with IC₅₀ values that were on par with the positive control used in DPPH and ABTS assays. This study highlights the potential medicinal value of LRH. Future studies should focus on the applications of the isolated compounds in health and conventional medicine or as natural antioxidants in the pharmaceutical and food sectors. Further studies may be required

to investigate sustainable cultivation and harvesting of LRH for its phytochemical components.

Keywords 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid · 2,2-diphenyl-1-picrylhydrazyl · Edible rose · Flavonoid · *Rosa hybrida* · Triterpenoid

Introduction

Natural products such as secondary metabolites derived from medicinal plants represent a significant and valuable resource in the therapeutic intervention of human physiological disorders and are excellent sources of novel pharmaceutical leads [1]. Specifically, terpenoids and flavonoids have been garnering attention over the past few decades due to their various biological activities. Further, they have been successfully employed as clinical pharmaceuticals [2-3]. More than 10,000 flavonoids have been successfully described, most of which are widely acknowledged for their therapeutic properties [4-5]. Moreover, structures of 60,000 terpenoids have been derived and identified from both plants and animals [6]. Similar to flavonoids, terpenoids exhibit a wide spectrum of biological activities and typically feature a cyclic structural configuration [7]. The biological efficacy of specific terpenoids is attributed to their lipophilic nature, which facilitates their partition into cellular membranes [8].

The genus *Rosa* from the Rose family (Rosaceae) comprises roughly 200 species [9]. These plants have been proven to harbor a wide array of natural products; hence, it is the plant of interest in this study. The rose has been grown as a garden plant for millennia and is one of the most popular ornamental plants in the world [10]. As they have long been used as cut flowers and in perfume, roses are a highly profitable crop in many countries. *Rosa hybrida* otherwise known as Scented Rose or Golden Rose, belongs to the Rosaceae family [11]. Aside from being popularly

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cultivated as an ornamental plant, members of this plant family are also used in various cuisines [12]. Aside from its ornamental and food uses, the Chinese have been using roses as a source of medicine [10]. European missionaries took Chinese rose specimens back to Europe with them. Since then, the genetic foundation of modern rose cultivars has been established through extensive hybridization between Chinese, European, Middle Eastern, and other wild *Rosa* species with extraordinarily diverse traits [13–14].

R. hybrida contains volatiles and other compounds with biomedical importance [15–16]. Owing to its wide array of phenolic compounds, *R. hybrida* has been previously reported to exhibit various bioactive properties like antitumor [17], antibacterial [18], anti-inflammatory [19], antidiabetic [20], and antioxidant [12] activities. However, no studies have used other parts of the plant like the roots and leaves, and these parts may contain the same compounds as the petals. These studies highlight the importance of *R. hybrida* in biomedical research.

Complete phytochemical profiling of this species has not been achieved yet. Hence, isolation, purification, and characterization of compounds from this plant are essential to elucidate its full potential as a medicinal plant [21]. Hence, this study aimed to isolate phytochemical constituents from *R. hybrida* and to identify its chemical structure. This study also investigated the antioxidant activity of *R. hybrida* extract using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS⁺) assays, coupled with total polyphenol and total flavonoid assays.

Material and Methods

Plant materials

The leaves of *R. hybrida* (LRH) were provided by Prof. Sung-Kwon Moon, Chung-Ang University, Republic of Korea. A voucher specimen of the plant was archived at the Department of Plant Science and Technology, Chung-Ang University, Anseong, Republic of Korea.

Chemicals and apparatus

Silica gel (200–400 mesh) from the American Society for Testing and Materials (Merck Co. in Darmstadt, Germany) served as the stationary phase for the open-column chromatography. The progress of chromatographic separations was tracked using thin-layer chromatography (TLC) plates precoated with silica gel 60 F254 (Merck KGaA, Germany, 0.25 mm). To visualize the compounds on TLC plates, they were sprayed with a mixture of 10% sulfuric acid in methanol (MeOH) and subsequently heated at 100°C on a hot plate. The solvents used for isolation, including *n*-hexane, chloroform (CHCl₃), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), and MeOH, were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea).

Total polyphenol and flavonoid content

The total polyphenol content (TPC) of LRH extracts was determined following a previously established method with minor modifications [22]. Specifically, 60 µL of each sample was mixed with 40 µL Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, 100 µL of 7.5% Sodium carbonate was added, and the samples were kept in the dark at room temperature for 30 min. The absorbance of the samples was then measured at 760 nm using a microplate reader (Epoch; BioTek, Winooski, VT, USA). The TPC was calculated based on a standard curve constructed using varying concentrations of tannic acid. Similarly, the total flavonoid content (TFC) of the samples was determined with a modified version of a previously described method [23]. In this case, 100 µL of 2% aluminum chloride hexahydrate was added to 100 µL of the extract and incubated for 10 min. The absorbance of the samples was measured at 430 nm using a microplate reader (Epoch; BioTek, Winooski, VT, USA). The TFC was calculated based on a standard curve constructed using varying concentrations of quercetin.

DPPH and ABTS⁺ assay

A working solution of 0.2 mM DPPH was prepared for the DPPH radical scavenging assay by diluting the DPPH stock solution with 95% ethanol (EtOH). Following this, 10 µL of the LRH extract was mixed with 200 µL DPPH working solution in 96-well plates. This was carried out in triplicates for accuracy. After thorough mixing using a microplate shaker, the reaction mixture was incubated in the dark for 30 min at room temperature. Then, its spectrophotometric absorbance was measured at 514 nm. Calculation of the DPPH radical scavenging rate facilitated the generation of calibration curves. For comparative purposes, ascorbic acid served as the standard. The ABTS⁺ solution was diluted with water to prepare the ABTS⁺ working solution. Subsequently, each test solution (10 µL) was added to the ABTS⁺ working solution (200 µL) in 96-well plates. This reaction was repeated three times to ensure precision. After thoroughly mixing in a microplate shaker, the reaction mixture was incubated for 30 min in the dark at room temperature. Post-incubation, the absorbance was measured at a wavelength of 734 nm. The calibration curve was established by calculating the ABTS⁺ radical scavenging rate. Ascorbic acid was used as the standard.

Extraction, fractionation, and isolation methods

The LRH (974 g) was subjected to EtOH extraction under reflux at 80 °C for 3 h, repeated 7 times. Following extraction, the solvents were filtered through filter paper and then evaporated using a rotary evaporator to yield an EtOH extract (193 g). This extract was subsequently suspended in water and divided into polarity fractions through sequential partitioning using *n*-hexane, CHCl₃, EtOAc, and *n*-BuOH. As a result, *n*-hexane (83 g), CHCl₃ (5 g), EtOAc (14 g), and *n*-BuOH (12 g) fractions were obtained.

Each fraction was further separated via open-column chromatography, using silica gel as the stationary phase. The EtOAc fractions were separated by eluting with a stepwise gradient of the CHCl_3 ·MeOH system. The EtOAc fraction yielded 6 sub-fractions. Sub-fractions 1-10 were recrystallized using MeOH to obtain a pure compound, and a single spot was identified in TLC. Sub-fractions 2, 3, 4, and 5 were identified as compounds **1**, **2**, **3**, and **4**, respectively.

Results and Discussion

The TPC and TFC of the LRH extracts were determined and analyzed. Table 1 summarizes the results of both assays. As shown in Table 1, the TPC of the extract was 45.51 mg/g, whereas the TFC was 5.672 mg/g. This may seem peculiar because flavonoids are a class of polyphenols [24]. However, assay plates are read at different wavelengths, and the wavelength selected for assessing the TPC (760 nm) is not adequately sensitive to detect specific flavonoids, which are better detected at different wavelengths (410 nm). Hence, variations are expected in results. Yang and Shin [12] conducted a similar study, wherein the TPC and TFC of nine *R. hybrida* spp. cultivars were evaluated. The nine samples had a TPC and TFC range of 798.67 to 2978.89 mg/100 g and 78.64 to 531.54 mg/100 g, respectively, which was relatively higher than that observed in the results of the present study. However, a recently published study by Kumari et al. showed a TPC range of 5.21 mg GAE/100 g FW (fresh weight) to 427.59 mg GAE/100 g FW per results obtained from 50 rose samples [25]. The differences in the results from these studies can be attributed to the light or dark coloration of petals used for extraction [26]. However, because the present study used leaves instead of petals, the differences between results can only be

speculated. Phenolic compounds may be concentrated in the floral parts of plants rather than in the leaves [15].

The antioxidant activity of the LRH extract was assessed using DPPH and ABTS⁺ radical scavenging assays (Table 2). In the DPPH assay, LRH exhibited significant scavenging activity at various concentrations (mg/mL). The percentages of scavenging activity at concentrations of 1.0, 0.50, and 0.25 mg/mL were found to be as follows: 63.52, 30.86, and 18.71%, respectively. The IC₅₀ value of the extract was 0.79 mg/mL, demonstrating an antioxidant efficacy comparable to that of ascorbic acid used as the standard, which exhibited an IC₅₀ value of 0.16 mg/mL. Furthermore, the scavenging activity of the LRH extract observed using the ABTS⁺ assay was assessed at 1.0, 0.50, and 0.25 mg/mL, yielding scavenging activity percentages of 6.25, 38.19, and 22.64%, respectively. The IC₅₀ value for this assay was determined to be 0.76 mg/mL, which was at par with the IC₅₀ value (0.12 mg/mL) of the standard, ascorbic acid.

Antioxidant capacities of plant extracts usually correlate with their polyphenol and flavonoid contents [27]. Some researchers conclude that the phenolic compounds in roses play a significant role in their antioxidant activity [28-29].

Four specific compounds were isolated from the LRH. The chemical structures of the compounds were determined using NMR spectroscopy, and the results were compared with those reported in previous studies (Fig. 1). The ¹H- and ¹³C-NMR data of compounds **1-4** are shown in Tables 3 and 4.

Compounds **1** and **2** were obtained as a white amorphous powder. The NMR spectra of compounds **1** and **2** indicated typical terpenoid structures (Table 3). Compound **1** exhibited a molecular ion peak at *m/z* 456 [M]⁺ (Supplementary Fig. 1). The ¹H-NMR spectrum of compound **1** showed peaks at δ_H 3.41 (1H, dd, *J*=11.5, 6.0 Hz) corresponding to H-3 and δ_H 5.48 (1H, br s) of the olefinic proton H-12. Furthermore, the ¹H-NMR spectrum shows the presence of methyl groups. For compound **1**, the methyl peaks can be identified at δ_H 1.32 (3H, s), 1.06 (3H, s), 0.94 (3H, s), and 1.06 (3H, s), corresponding to protons number 23-27 and 29-30, respectively. These peaks suggest that the structure of the compound is similar to that of an olean-12-ene-

Table 1 The TPC and TFC of LRH

Sample	TPC (mg TAE/g extract)	TFC (mg QE/g extract)
LRH	45.51±3.128	5.672±3.374

Note: TAE, tannic acid equivalent; QE, quercetin equivalent

Table 2 Radical scavenging activity of LRH extracts determined using the DPPH and ABTS⁺ assays

Sample	Concentration (mg/mL)	DPPH		ABTS ⁺	
		Scavenging activity (%)	IC ₅₀ (mg/mL)	Scavenging activity (%)	IC ₅₀ (mg/mL)
LRH	1.0	63.52±3.90	0.79±0.06	6.25±2.47	0.76±0.06
	0.50	30.86±3.78		38.19±7.75	
	0.25	18.71±2.36		22.64±4.73	
AA	0.2	68.102±0.863	0.16±0.00	86.09±1.47	0.12±0.00
	0.16	48.438±4.045		63.58±0.55	
	0.12	36.547±0.863		49.29±0.18	
	0.08	17.264±0.349		27.79±0.31	
	0.04	1.067±0.959		15.27±0.10	

Note: AA, ascorbic acid as a positive control

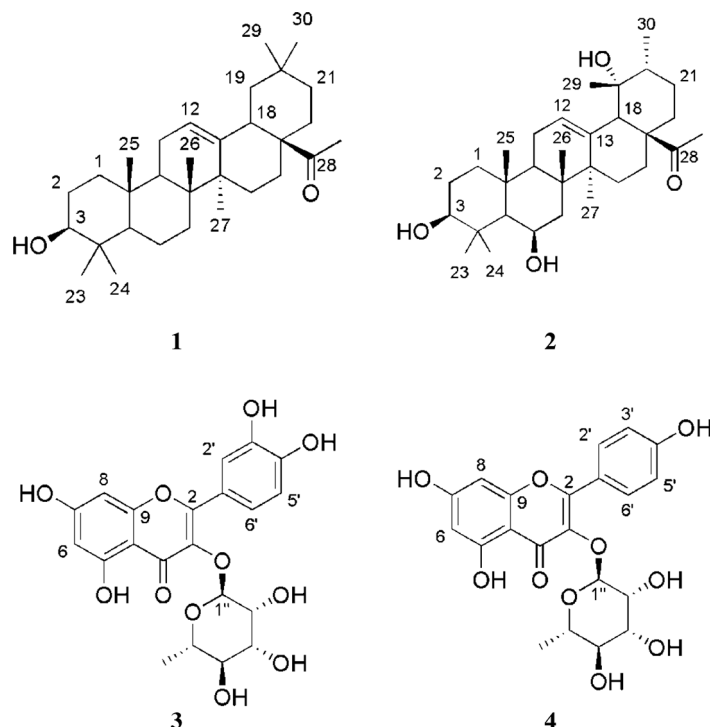


Fig. 1 Chemical structures of oleanolic acid (1), uncaric acid (2), quercetin 3-*O*-rhamnoside (3), and kaempferol 3-*O*-rhamnoside (4)

type triterpenoid. The ^{13}C -NMR spectrum of compound **1** showed two olefinic peaks at δ_{C} 123.0 and 145.3, one carboxylic acid peak at δ_{C} 180.4, seven methyl signals at δ_{C} 28.8, 17.5, 17.3, 17.7, 25.4, 34.0, and 21.9 (Supplementary Fig. 2). Analysis of these peaks together with spectral data comparison with previous literature confirm that compound **1** is oleanolic acid [30].

Compound **2** exhibited a molecular ion peak at m/z 488 $[\text{M}]^+$ (Supplementary Fig. 3). The ^1H -NMR spectrum of compound **2** showed peaks at δ_{H} 3.40 (1H, d, $J=9.0$ Hz) corresponding to H-3 and δ_{H} 5.60 (1H, s) of the olefinic proton H-12. Furthermore, the ^1H -NMR spectrum shows the presence of methyl groups. For compound **2**, the five methyl peaks can be identified at δ_{H} 1.09 (3H, s), 1.14 (3H, s), 1.36 (1H, s), 1.12 (3H, s), 1.34 (3H, s), and 1.25 (3H, s) while the only doublet methyl peak can be found at δ_{H} 1.03 (3H, d, $J=7.5$ Hz), corresponding to protons number 23–27 and 29–30, respectively. These peaks suggest that the structure of the compound is similar to that of an urs-12-ene-type triterpenoid. The ^{13}C -NMR spectrum of compound **2** showed two olefinic peaks at δ_{C} 123.0 and 145.3, one carboxylic acid peak at δ_{C} 181.1, seven methyl signals at δ_{C} 29.7, 17.9, 17.7, 18.2, 24.6, 27.4, and 17.3 (Supplementary Fig. 4). Analysis of these peaks together with spectral data comparison with previous literature confirm that compound **2** is uncaric acid [31].

Terpenoids are another class of terpenes that contain oxygen molecules [32]. Terpenoids are the largest class of secondary metabolites found in plants with complex biosynthesis and regulation [33]. Some types of terpenoids are alcohols, aldehydes,

esters, ethers, epoxides, ketones, and phenols [34]. Terpenoids play a significant role in how plants, microbes, and animals interact and defend themselves from various threats [35]. Oleanolic (**1**) and uncaric (**2**) acids are triterpenoids that have importance in the pharmaceutical field, as they have various biological activities such as anticancer [36], anti-inflammatory [37], and antioxidant properties [38].

Compounds **3** and **4** were obtained as a yellow amorphous powder. Compounds **3** and **4** were confirmed to be flavonoid glycosides according to their ^1H -NMR spectrum exhibiting aromatic proton signals and a hydroxy proton at position 5 (Table 4). Compound **3** exhibited a molecular ion peak at m/z 302 $[\text{M-rhamnoside}]^+$ (Supplementary Fig. 5). The ^1H -NMR spectrum of **3** also exhibited signals at δ_{H} 7.32 (1H, d, $J=2.0$ Hz), 6.88 (1H, d, $J=8.5$ Hz), and 7.26 (1H, dd, $J=8.5, 2.0$ Hz) attributable to the ABX coupling system of the B ring structure. The anomeric proton resonance, observed at δ_{H} 5.26 (1H, d, $J=1.0$ Hz) corresponded to the presence of the glucose moiety (Supplementary Fig. 6). On the other hand, compound **4** exhibited a molecular ion peak at m/z 286 $[\text{M-rhamnoside}]^+$ (Supplementary Fig. 7). The ^1H -NMR spectrum of compound **4** exhibited signals at δ_{H} 7.75 (2H, d, $J=9.0$ Hz, H-2',6') and 6.92 (2H, d, $J=8.5$ Hz, H-3',5') attributable to the symmetrical A_2B_2 coupling system of the B ring structure. The anomeric proton resonance, which occurred at δ_{H} 5.29 (1H, d, $J=1.0$ Hz) corresponded to the presence of the glucose moiety (Supplementary Fig. 8). Consequently, compounds **3** and **4** were identified as quercitrin (quercetin 3-*O*-rhamnoside), and afzelin

Table 3 ^1H - and ^{13}C -NMR data for compounds **1** and **2**

No.	1		2	
	δ_{H}	δ_{C}	H	δ_{C}
1a	-	38.9	-	40.9
1b	-	-	1.45-1.39 (2H, m)	-
2a	-	27.4	1.51-1.49 (1H, m)	29.1
2b	-	-	1.73-1.58 (5H, m)	-
3	3.41 (1H, dd, 11.5, 6.0)	73.2	3.40 (1H, d, 9.0)	84.3
5	-	55.1	1.0 (1H, d, 4.5)	40.3
6	-	18.2	5.07 (1H, s)	56.4
7a	-	31.6	1.45-1.39 (2H, m)	69.1
7b	-	-	1.73-1.58 (5H, m)	-
8	-	39.3	-	37.9
9	-	48.1	1.73-1.58 (5H, m)	48.8
10	-	37.9	-	34.0
11	-	21.9	2.00-1.96 (2H, m)	24.4
12	5.48 (1H, br s)	123.0	5.60 (1H, s)	128.4
13	-	145.3	-	140.4
14	-	40.9	-	42.8
15a	-	27.6	1.90 (1H, br.s)	29.8
15b	-	-	1.06 (1H, s)	-
16a	-	22.8	3.15 (1H, ddd, 13.3, 13.3, 4.2)	26.8
16b	-	-	1.69-1.58 (2H, m)	-
17	-	46.9	-	54.0
18	-	40.5	2.36-2.35 (1H, m)	55.1
19	-	47.1	-	73.2
20	-	31.5	1.45-1.39 (1H, m)	42.6
21	-	33.8	-	27.6
21b	-	-	1.73-1.58 (5H, m)	-
22a	-	33.7	1.69-1.58 (2H, m)	39.0
22b	-	-	1.73-1.58 (5H, m)	-
23	1.32 (3H, s)	28.8	1.09 (3H, s)	29.7
24	1.06 (3H, s)	17.5	1.14 (3H, s)	17.9
25	1.03 (3H, s)	17.3	1.36 (1H, s)	17.7
26	0.98 (3H, s)	17.7	1.12 (3H, s)	18.2
27	1.28 (3H, s)	25.4	1.34 (3H, s)	24.6
28	-	180.4	-	181.1
29	0.94 (3H, s)	34.0	1.25 (3H, s)	27.4
30	1.06 (3H, s)	21.9	1.03 (3H, d, 7.5)	17.3

Chemical shifts were reported in parts per million (δ) and coupling constants (J) were expressed

(kaempferol 3-*O*-rhamnoside), respectively, by comparing the data of authentic compounds with existing literature [39–40].

Similar to terpenoids, flavonoids constitute a significant group of natural products, characterized by a polyphenolic structure, and are commonly found in various fruits and vegetables [41]. Flavonoids play a crucial role in diverse biological processes within plants [42]. They contribute to the color and fragrance of flowers and are responsible for attracting pollinators [43]. This, in

turn, facilitates the dispersion of fruits and aids in seed and spore germination, as well as the growth and development of seedlings [44]. Additionally, flavonoids often serve as ultraviolet (UV) screens and act as antioxidant agents, providing protection to plants against UV radiation and various biotic and abiotic stressors [45–46]. Flavonoids like quercitrin (**3**) and afzelin (**4**) have long been associated with a broad spectrum of biological activities, such as anti-inflammatory [47], anticancer [48], and antibacterial

Table 4 ^1H - and ^{13}C -NMR data for compounds **3** and **4**

No.	3		4	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
2	-	156.4	-	157.3
3	-	134.2	-	134.2
4	-	177.7	-	178.7
5	-	161.3	-	163.2
6	6.22 (1H, d, 2.0)	98.9	6.20 (1H, d, 1.5)	98.9
7	-	164.4	-	163.2
8	6.42 (1H, d, 2.0)	93.7	6.41 (1H, s)	93.8
9	-	157.3	-	156.5
10	-	104.0	-	108.4
1'	-	121.1	-	120.5
2'	7.32 (1H, d, 2.0)	115.5	7.75 (2H, d, 9.0)	115.4
3'	-	145.2	6.92 (2H, d, 8.5)	130.6
4'	-	148.5	-	159.9
5'	6.88 (1H, d, 8.5)	115.6	6.92 (2H, d, 8.5)	115.4
6'	7.26 (1H, dd, 8.5, 2.0)	120.7	7.75 (2H, d, 9.0)	130.6
1''	5.26 (1H, d, 1.0)	101.8	5.29 (1H, d, 1.0)	101.7
2''	3.52 (1H, dd, 9.5, 3.3)	70.4	4.20 (1H, dd, 14.3, 7.3)	70.3
3''	3.26-3.20 (1H, m)	70.6	4.08 (1H, dd, 14.0, 7.0)	70.6
4''	3.17-3.14 (1H, m)	71.2	3.15-3.08 (2H, m)	71.1
5''	3.99 (1H, dd, 3.0, 1.5)	70.1	3.15-3.08 (2H, m)	70.1
6''	0.82 (3H, d, 6.5)	17.5	0.79 (3H, d, 6.0)	17.4
5-OH	12.66 (s)	-	12.62 (s)	-

Chemical shifts were reported in parts per million (δ) and coupling constants (J) were expressed

[49] activities, and their ability to modulate the functions of key cellular enzymes [37].

The presence of both terpenoids and flavonoids can be linked to the robust antioxidant activity of the LRH extract, as shown in both the DPPH and ABTS⁺ assays. Notably, previous studies were primarily focused on elucidating the antioxidant properties of the floral components of this plant, with limited attention given to those of the leaves. Taken together with the existing reports, the results of this study highlight the importance of each part of a plant and aid in understanding its value as a potential ingredient in pharmaceutical and nutraceutical products. This study also highlights the importance of phytochemical profiling of plants, as it can give researchers a general idea of the biological activities of a plant [50].

The results of this study will facilitate the phytochemical profiling of LRH and plants in general. This study also serves as a reference for future studies in this field. Finally, this study can improve the market value of LRH in various industries.

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