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Research Article

FREE RADICAL SCAVENGING ACTIVITY OF THE STANDARDIZED ETHANOLIC EXTRACT OF *EURYCOMA LONGIFOLIA* (TAF-273)

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ABSTRACT

Eurycoma longifolia is widely used as traditional herbs, especially in Indonesia, Malaysia, and Vietnam. Many studies have been published regarding the anti-plasmodial activity, cytotoxic effect, antipyretic, induce apoptosis in HepG2 cells and aphrodisiac property. In one study, it was claimed that *E. longifolia* extract may promote healthy aging in man. To date, no results has been published on determination of its phenolic content or free radical scavenging activity. This study was performed to investigate the free radical scavenging activity of the standardized ethanolic extract of *Eurycoma longifolia* (TAF-273) related with the total phenolic and flavonoid contents, and the gas chromatography-mass spectrometry (GC-MS) analysis. Free radical scavenging activity of extract was determined by DPPH method with some modifications at 517 nm by using a microplate reader. Phenolic content was determined by Folin-Ciocalteu reagent at 765 nm and the flavonoid content was determined by aluminum chloride colorimetric method at 415 nm. GC-MS analysis was done by using an Agilent GC-MS. TAF-273 contained 17.142±1.102 µg GAE/mg dry weight of phenolic content and 0.253±0.016 µg QE/mg dry weight of flavonoid content. The phenolic compounds in TAF-273 detected by using GC-MS analysis were identified as 2,6–dimethoxy-phenol (3.03%), 2-methoxy-4-(1-propenyl)-(E)-phenol (1.08%), 3,4,5-trimethoxy-phenol (1.60%), 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (1.65%) and 4-(2,3-dihydro-7-methoxy-3-methyl-5-propyl-2-benzofuranyl)-2-methoxy-phenol (2.47%). The free radical scavenging activity of TAF-273 was in a linier correlation with the increase of its phenolic content. The activity of TAF-273 (EC_{50} = 754 µg/mL) was lower as compared with standard of gallic acid (EC_{50} = 3.035 µg/mL).

Keywords: Free radical scavenging activity, Phenolic content, Flavonoid content, *Eurycoma longifolia,* GC-MS

INTRODUCTION

One of the traditional medicine that has been widely used, especially in Indonesia, Malaysia and Vietnam to cure erectile dysfunction (ED) is *Eurycoma longifolia* Jack (*E. longifolia*)[1]. Many studies have been published in regards with the anti-plasmodial activity, cytotoxic effect, antipyretic, antidiabetic activity and the interaction study[2-6]. There are many publications available on the aphrodisiac properties. The effects of *E. longifolia* roots extracts (chloroform, butanol, methanol and water) on the libido of sexually experienced male rats was reported by Ang and Sim (1997)[7]. Ang and Ngai (2001)[8] evaluated the aphrodisiac effect of fractions of *E. longifolia* roots in non-copulator male rats using an electrical cage. Ang and Lee (2002)[9] provided evidence pertaining to changes in sexual behavior (orientation activities) among the middle-aged male rats after administering different fractions of the *E. longifolia* root extracts. One study by Ang *et al.* (2003)[10] reported that administering *E. longifolia* root extracts affected the sexual motivation activity in adult, middle-aged male mice and in retired breeders.

In one study, it was claimed that *E. longifolia* extract may promote healthy aging in man. Dr Ismail Tambi (2009)[11] conducted a double-blind placebo controlled human clinical trial, 20 male volunteers that were randomly given 200, 400 or 600 mg of standardized water extract or placebo for 2 months showed an improvement of Aging Male Score (AMS). It showed 91% improvement of sexual score, in the physical score (73%), 91% and 52% improvement of the psychological score and in vasomotor score, respectively. Phenolic compounds are secondary metabolites which synthesize in plants. They possess biological prosperities such as: anti-oxidant, anti-apoptosis and anti-aging[12]. Oxidation is an important process for living organisms. The uncontrolled production of oxygen free radicals and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and aging. Therefore, phenolic content of substance is usually related with anti-oxidant and anti-aging capacity. To date, no results (very limited data) has been published on determination of phenolic content or free radical scavenging properties of *E. longifolia*. Present study evaluated the free radical scavenging activity of the standardized ethanolic extract of *E. longifolia*.

MATERIALS AND METHODS

Plant material

The standardized ethanolic extract of *E. longifolia* (TAF-273) was made and standardized in Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, University Sains Malaysia following the protocol described previously and the voucher specimen of the plant was deposited with Reference No. 785-117 at Penang Botanical Garden[1]. The extract was standardized by using euryomanone as a marker compound, it contained 19.6% of eurycomanone[13].

Chemicals

Quercetin and gallic acid standard were purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium hydrogen carbonate was purchased from BDH Laboratory Supplies, UK, while potassium acetate, aluminum chloride, Folin-Ciocalteu reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals (St. Louis, MO, USA). Methanol of HPLC grade was obtained from Merck (Darmstadt, Germany).

Determination of total flavonoid content

The aluminum chloride colorimetric method was used to determined the total flavonoid content of TAF-273 and was modified from the procedure reported by Woisky and Salatino (1998)[14]. Quercetin was used to make the calibration curve. It was made in a serial concentration from 33.3 to 500 µg/mL. The standard compound or sample was dissolved in methanol, the solution was taken out (0.5 mL) and was placed in the test tube. Then, it was added with 0.1 mL of 10% (w/v) aluminum chloride, potassium acetate (1 M; 0.1 mL) and methanol (1.5 mL). Finally, distilled water (2.8 mL) was added to this mixture. The solution was mixed well and the absorbance was determined by using a microplate reader (Power wave X-340®, Biotek) at 415 nm after incubation for 30 min at room temperature. Results were expressed as µg quercetin equivalent per mg dry weight of extract (µg QE/mg d.w.).

Determination of total phenolic content

The total phenolic content of TAF-273 was determined by the Folin-Ciocalteu method with some modifications[15]. The extract or standard in methanol (50 µL) was oxidized with Folin-Ciocalteu reagent (375 µL;10 times dilution of 2 N) for 5 min, and the reaction was neutralized by adding 6% (w/v) sodium carbonate (375 µL). The absorbance of the resulting dark blue color was measured at 765 nm after 90 min incubation at room temperature. Gallic acid was used as standard of total phenolic content. It was made by a serial dilution of gallic acid at concentrations 50 to 800 µg/mL. Results were expressed as µg gallic acid equivalent/mg dry weight of extract (µg GAE/mg d.w.).

Determination of DPPH scavenging activity

The free radical scavenging activity of TAF-273 and standard of gallic acid were measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH that was described by Kumaran & Karunakaran (2006)[16] with some modifications. Therefore, solution standard was made by dissolving of gallic acid in methanol at concentrations 0.32 to 5 µg/mL. TAF-273 was dissolved in the same solvent and was diluted at concentrations 0.063 to 4 mg/mL. DPPH solution (100 µL; 0.23 mM) was filled in 96-well plate and sample or standard solution (100 µL) was added. After 30 min of incubation at 37ºC, the absorbance was measured at 517 nm by using a microplate reader. A control contained 100 µL DPPH solution and 100 µL methanol as the blank. Free radical scavenging activity of the extract and standard of gallic acid were determined according to the following formula:

$$\text{Free radical scavenging activity (\%)} = [1 - (As/Ac)] \times 100$$

Where A*s* is the absorbance of test sample and *A*c is the absorbance of control,

All data in this study were expressed as mean ± SD (n=3), unless for GC-MS analysis results.

Gas chromatography-mass spectrometry (GC-MS) analysis

Characterization of the unknown chemicals of TAF-273 was performed using an Agilent GC mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with flame ionization detection (FID). It consists of a 6890 GC system, a 7683 series injector and a quadruple mass analyzer model 5973. Sample was prepared by dissolving 20 mg TAF-273 in 2 ml methanol. The oven was set at an initial temperature 70ºC and 280ºC as final temperature, at ramping rate of 20ºC/min, using a splitless injection with injector temperature was fixed at 280ºC. The running time was 42.50 min. Helium was used as carrier gas at a flow rate of 1.2 mL/min using a HP-5MS capillary column (30 m X 0.25 mm X 0.25 µm). The ion source temperature was maintained at 250ºC with the mass range of detection configured between 33-650 amu. The spectral scan rate was set at 20 spectra/s and the volume of sample injection was 1 µL. Data analysis of peaks observed was acquired via the Waters Millenium 32 software and identification of the compounds was carried out by referring to NIST (National Institute for Standard and Technology) 2002 Mass Spectral Database and the Terpene Essential Oil Library[17].

Statistical analysis

The correlation between total phenolic content in TAF-273 and the free radical scavenging activity was analyzed by using Pearson correlation test. The P-value less than 0.01 was considered statistically significant.

RESULTS AND DISCUSSION

There are some factors that are able to accelerate an aging process, such as imbalance of body defense system and polluted environment. These factors cause oxidative stress. Oxidative stress results in oxidative alteration of biological macromolecules such as lipids, proteins and nucleic acids. It is considered to play a pivotal role in the pathogenesis of aging and degenerative diseases. Human bodies have developed sophisticated mechanisms for maintaining redox homeostasis to protect the effect of free radicals produced upon oxidative stress. These protective mechanisms include scavenging or detoxification of reactive oxygen species. Antioxidants through their activity contribute to the protection of organism against oxidational stress[18,19]. Many herb-drugs contain natural anti-oxidants such as pholyphenols, flavonoids and phenolic compounds that protect cells from the oxidative stress effect[20].

Determination of total flavonoid and phenolic content

The quantitative analysis of total flavonoid content of TAF-273 was performed by using aluminum chloride colorimetric method at 415 nm and used quercetin as standard of total flavonoid. While, Folin-Ciocalteau method at 765 nm was used to determine its total phenolic content with gallic acid as a standard. The linier equation of quercetin and gallic acid standard curve were Y = 0.004 X – 0.011; R^2 = 0.999 and Y = 0.003X – 0.009; R^2 = 0.996, respectively. Where X was the quercetin or gallic acid concentrations in µg/mL and Y was the absorbance at 415 nm or 765 nm. TAF-273 contained the total flavonoid of 0.253±0.016 µg QE/mg d.w. and the phenolic compounds of 17.142±1.102 µg GAE/mg d.w. Colorimetric methods targets flavonoids of similar structures are convenient and appropriate for routine analyses. However, none of the colorimetric methods can detect all kinds of flavonoids. This method was proved to be specific only for flavones and flavonols[21,22].

Flavonoids is one of the phenolic compounds groups and the most abundant polyphenols in human diets. The phenolic compounds are believed to have the ability of antioxidant because of rich in hydroxyl groups. Polyphenols are multifunctional and can act as reducing agents, as hydrogen atom–donating antioxidants, chelating metal ions and as singlet oxygen quenchers. They have been reported to possess potent anti-oxidant activity by different mechanism such as scavenging free radicals, inhibiting oxidant enzymes, impacting cell cycle, inducing endogenous anti-oxidant enzymes or modulation of signals transduction[12]. Therefore, this study needed to determine the flavonoid and phenolic content of TAF-273 before determining the free radical scavenging activity. It was found that the flavonoid content in TAF-273 (0.025 ±0.002%) was lower than the phenolic content (1.714±0.110%).

Determination of DPPH scavenging activity

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[23,24]. The reduction in DPPH radical was determined by the decrease of its absorbance at 517 nm by anti-oxidants. One experiment was done before the determination of DPPH scavenging activity of TAF-273, to know whether there is any correlation between TAF-273 concentrations and the total phenolic content. The result showed that there was an increase of total phenolic content in the increasing of TAF-273 concentrations (Fig. 1).

In this study gallic acid was used as a standard. Figs. **2** and **3** show the correlation between scavenging activity (%) with gallic acid and TAF-273 concentrations, respectively. Based on the Pearson correlation test results, the total phenolic content in TAF-273 has a linier correlation with the percentage of free radical scavenging activity (P<0.01). The EC_{50} of TAf-273 and gallic acid standard were 754 µg/mL and 3.035 µg/mL, respectively. The phenolic content of TAF-273 was only 1.714%, led to lowering of the free radical scavenging activity. It was almost 250 times lower than standard of gallic acid. Even though the phenolic content was low, it may have a potential effect as anti-aging if it was related with Dr Tambi's study.

GC-mass spectrometry analysis

Fig. 4 shows the results of the GC-MS chromatogram of TAF-273. The components in the TAF-273 were detected at the retention time in the range from 7 to 17 min. The total components detected were 31 types. Table 1 reveals the identified molecular components that have a matching library of≥90%. There were 10 components and some of them were phenolic compound: 2,6–dimethoxy-phenol (3.03%), 2-methoxy-4-(1-propenyl)-(E)-phenol (1.08%), 3,4,5-trimethoxy-phenol (1.60%) and 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (1.65%).

Table **2** shows the other detected components that have a matching library <90%. There were three components have a high abundance

that were detected at retention time 12.60, 12.64 and 13.53 min and were identified as 2,3-dihydro-2-hydroxymethyl-4,8-dimethyl-furo[3,2-c]quinoline (10.20%), 4-methoxy-4',5'-methylene dioxybiphenyl -2-carboxylic acid (15.96%) and 9,10-didehydro-6-methyl- (8.beta.)-ergoline-8-methanol (6.60%), respectively. One of phenolic compounds was detected at retention time 12.21 min and was identified as 4-(2,3-dihydro-7-methoxy-3-methyl-5-propyl-2-benzofuranyl)-2-methoxy-phenol (2.47%).

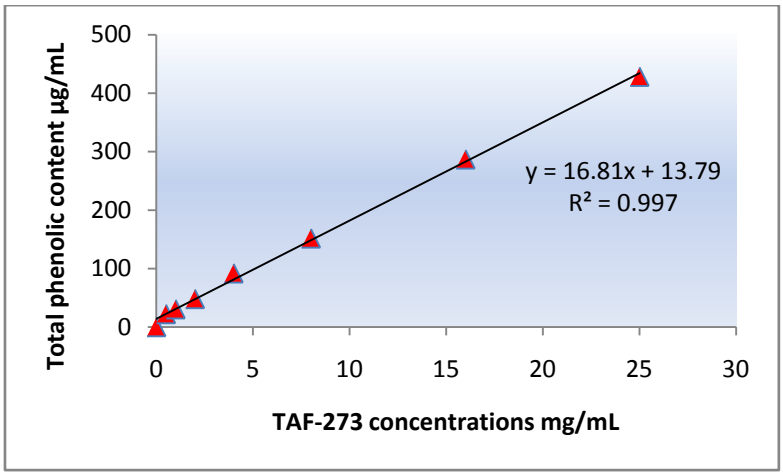

Fig. 1: Linier correlation between TAF-273 concentrations and its phenolic content

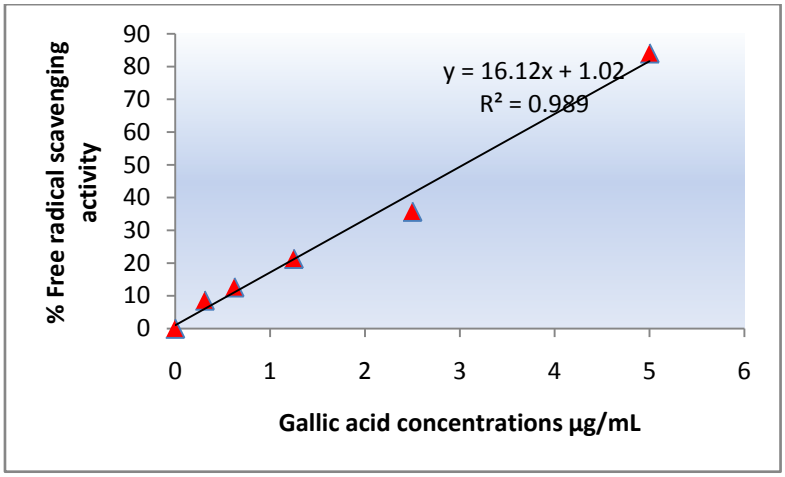

Fig. 2: Free radical scavenging activity of gallic acid

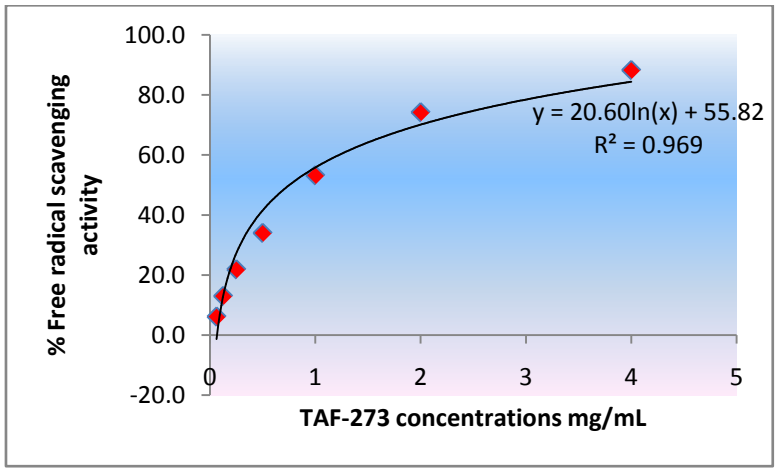

Fig. 3: Free radical scavenging activity of TAF-273

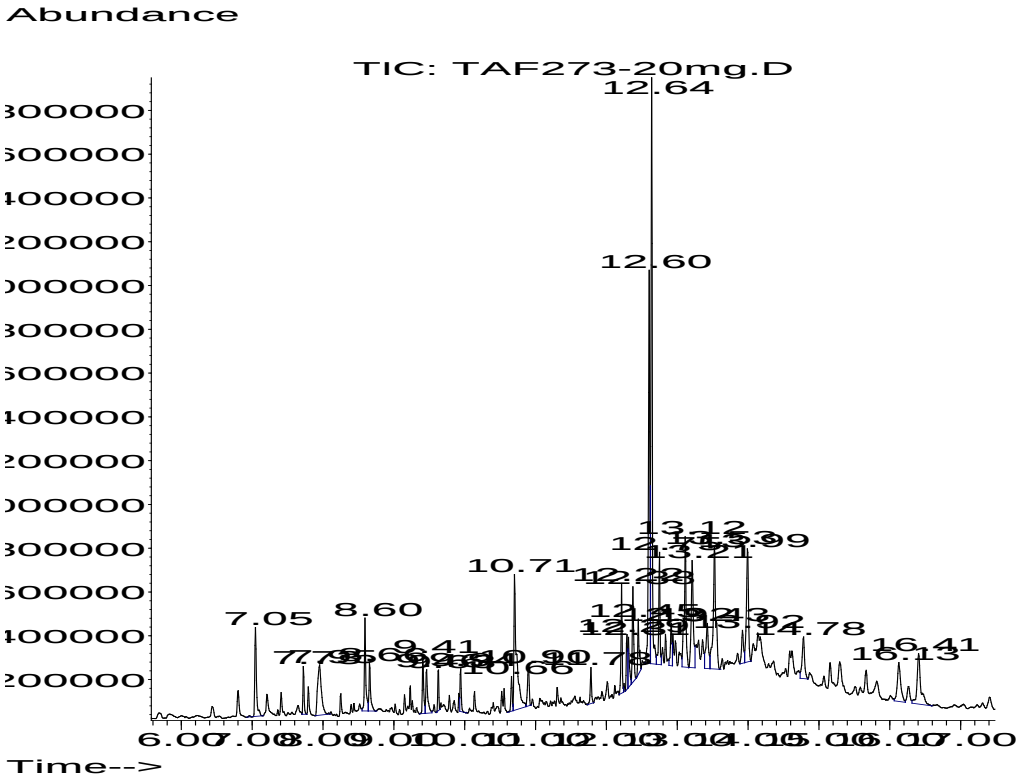

Fig. 4: GC-MS chromatograms of TAF-273

Table 1: The identified phytochemicals in the TAF-273 were detected using GC-MS (matching library of ≥90%)

Peak No	Phytochemicals	Molecular Formula	Retention Time (min)	Area (%)
1	Phenol, 2,6 –dimethoxy-	C8H10O3	7.05	3.03
2	Phenol, 2-methoxy-4-(1-propenyl)- (E)-	C10H12O2	7.73	1.08
3	1,6-Anhydro-.beta.-D-glucopyranose (levoglucosan)	C6H10O5	7.95	3.85
4	2,4'-Dihydroxy-3'-methoxyacetophenone	C9H10O4	8.59	2.61
5	Phenol, 3,4,5-trimethoxy-	C9H12O4	8.66	1.60
6	Benzeneacetic acid, 4-hydroxy-3-methoxy-, methyl ester	C10H12O4	9.41	1.53
7	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	C10H12O3	9.46	1.65
11	7-Hydroxy-6-methoxy-2H-1-benzopyran-2-one	C10H8O4	10.71	7.13
28	3,7-Dibromo-2-hydroxy-5-methoxymethyl-2,4,6-cycloheptatrien-1-one	C9H8Br2O3	14.00	4.74
31	1,5,7,11-Ethanediylidene-3,5:9,11-dimethanochrysene, hexadecahydro-	C22H28	16.41	3.77

Table 2: The identified phytochemicals of TAF-273 were detected using GC-MS (matching library of <90%)

Peak No	Phytochemicals	Retention Time (min)	Area (%)
8	3,5-Dimethoxy-4-hydroxyphenylacetic acid	9.63	0.90
9	Carbazole	9.94	1.11
10	Benzaldehyde, 4-[[4-(acetyloxy)-3,5-dimethoxyphenyl]methoxy]-3-methoxy-	10.66	0.88
12	Benzene, 1-ethyl-3,5-diisopropyl-	10.90	1.36
13	2(1H)-Quinolinone, 4-phenyl-	11.78	0.91
14	Phenol, 4-(2,3-dihydro-7-methoxy-3-methyl-5-propyl-2-benzofuranyl)-2-methoxy-	12.21	2.47
15	7H-Furo[3,2-g][1]benzopyran-7-one,2,3,5-trimethyl-	12.29	1.30
16	Naphtho[2,1-b]furan-4-ol, 1,2-dihydro-2-methyl-5-(2-propenyl)-	12.31	1.43
17	Azacyclotridecan-2-one, 1-(3-aminoprophyl)-	12.37	2.67
18	2,4-Diamino-5-benzyl-6-methylthieno[2,3-d]pyrimidine	12.45	1.51
19	Furo[3,2-c]quinoline, 2,3-dihydro-2-hydroxymethyl-4,8-dimethyl-	12.60	10.20
20	.alpha.-Estradiol	12.64	15.96
21	Cobalt, [(1-buthyl)cyclopentadienyl]-1,2-dithiolatoethene	12.75	2.88
22	.alpha.-Estradiol	12.92	1.23
23	Silane, (estra-1,3,5(10),16-tetraen-3-yloxy)trimethyl-	13.12	4.90
24	Benzoic acid, 2-[2-(2,3-dimethoxyphenyl)ethenyl]-	13.22	4.44
25	Medazepam	13.42	2.32
26	Ergoline-8-methanol, 9,10-didehydro-6-methyl- (8.beta.)-	13.53	6.60
27	4H-Naphtho[2,3-b]pyran-4-one, 5,6-dihydroxy-8-methoxy-2-methyl-	13.93	1.23
29	1-Methoxyestrone	14.78	2.08
30	1-Anilinobicyclo(3.2.0)hept-3-en-2-one	16.13	2.63

CONCLUSION

TAF-273 contained the total flavonoid of 0.253±0.016 µg QE/mg d.w. and the phenolic compounds of 17.142±1.102 µg GAE/mg d.w. The phenolic compounds in TAF-273 detected by using GC-MS analysis were identified as 2,6–dimethoxy-phenol (3.03%), 2-methoxy-4-(1-propenyl)-(E)-phenol (1.08%), 3,4,5-trimethoxy-phenol (1.60%), 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol (1.65%) and 4-(2,3-dihydro-7-methoxy-3-methyl-5-propyl-2-benzofuranyl)-2-methoxy-phenol (2.47%). The free radical scavenging activity of TAF-273 (EC_{50} = 754 µg/mL) was lower as compared with standard of gallic acid (EC_{50} = 3.035 µg/mL).

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