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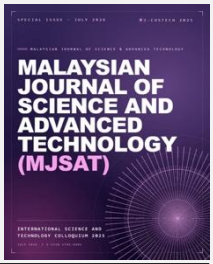

Antioxidant And Antiaging Potentials of *Justicia Gendarussa* Leaf and Root Methanolic Extracts

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ABSTRACT

Premature and accelerated skin aging is a complex skin complication affecting millions worldwide. Excessive exposure to ultraviolet radiation causes reactive oxygen species accumulation that promotes premature skin aging. Topical skincare application is a common therapeutical method but may contain dangerous chemicals. Thus, plant-based skin products are gaining prominence among consumers. *Justicia gendarussa* is a local plant traditionally used to treat multiple conditions. However, the antiaging property of this plant is still unclear. The aim of this study was to extract phytochemicals from the leaf and root of *J. gendarussa* and assess their antioxidant and antiaging potentials. Total phenolic and flavonoid content were measured using colorimetric assays. DPPH radical scavenging and enzyme inhibition assays were used to determine the antioxidant and antiaging properties of the extracts. *J. gendarussa* leaf (JGL) extract showed a higher TPC value but slightly lower TFC value than the root (JGR) extract. Both extracts showed dose-dependent DPPH radical scavenging activities with maximum inhibitions of about 40% at 0.5 mg/ml concentration. The leaf extract demonstrated higher (47%) anti-tyrosinase activity than the root extract (37%). Both extracts showed low anti-collagenase activities. Nonetheless, this study suggests *J. gendarussa* possesses antioxidant and antiaging properties and highlights its potential application in developing novel natural-based skincare formulations.

1. INTRODUCTION

Skin is the largest organ, with its principal role as the primary physical barrier to protect internal tissues and organs from external insults. The skin contains several different types of cells, such as keratinocytes, melanocytes, fibroblasts, and others, that are arranged in three main layers, namely, epidermis, dermis, and hypodermis. Over time, exposure to numerous intrinsic and extrinsic factors causes skin cell death and alters matrix composition, compromising the structural integrity of the tissue [1]. While skin can naturally age, premature and accelerated aging typically results in prominent clinical manifestations such as deep wrinkles, sagging skin, hyper- and uneven pigmentation, dryness, and many others [2].

At the molecular level, skin aging involves a myriad of factors with reactive oxygen species (ROS) as the central mediator [3]. Extrinsic factors such as UV radiation have been shown to cause ROS accumulation that in turn activates various signaling pathways involving mitogen-activated protein kinase (MAPK), microphthalmia-associated transcription factors (MITF), and other molecular factors [3, 4]. Aberrant activation of these factors elevates the expression of tyrosinase, collagenase, elastase, and other enzymes that contribute to the clinical presentation of skin aging [5].

Justicia gendarussa, locally known as "Genda Rusa", is a small shrub species from the Acanthaceae family [6]. This plant contains numerous bioactive compounds, including steroids, alkaloids, flavonoids, polyphenols, saponins, and essential oils [6, 7]. *J. gendarussa* has been reported to demonstrate numerous pharmacological properties such as antioxidants, anticancer, antiviral, anti-inflammation, antibacterial, and

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many other properties [7, 8]. The reported IC_{50} of DPPH radical scavenging of *J. gendarussa* extracts range from 0.075-0.223 mg/ml, indicating a strong antioxidant activity [9, 10]. Patel et al. reported IC_{50} values ranging from 0.0052-1564 µg/ml of collagenase inhibition for methanolic leaf extract and various solvent fractions [11]. However, other antiaging effects of *J. gendarussa*, namely tyrosinase inhibition potential, have not been demonstrated previously. Given its rich phytochemical profile and traditional uses, the aim of this study was to evaluate the antioxidant and antiaging properties of the leaf and root methanolic extracts of *J. gendarussa*. Both extracts showed DPPH radical scavenging activities. Tyrosinase inhibition was moderate but reflects the novelty of the study. Both extracts demonstrated low collagenase inhibition.

2. MATERIALS AND METHODS

2.1 Extraction of Justicia gendarussa

Leaves and roots of *J. gendarussa* were procured from a local market in Kangar, Perlis. Upon collection, the samples were weighed, thoroughly washed with distilled water to remove impurities and subsequently air dried. Once fully dried, the leaves and roots were ground into fine powder using a mechanical blender, yielding 20 g of powdered material.

Soxhlet extraction was employed for the extraction process, using absolute methanol as the solvent. A sample to solvent ration of 1:10 was used, with 200 mL of methanol per 20 g of plant powder. The extraction was carried out at 65°C for 12 hours, with multiple cycles repeated until the solution in the extractor turned colorless, indicating the exhaustion of extractable compounds. Due to the limited amount of the powdered samples, only one Soxhlet extraction was performed for the leaf and root samples.

Following extraction, the methanolic extracts were concentrated using a rotary evaporator at 62°C to remove excess solvent and obtain the crude extracts. The extracts were left air-dried in the fume hood for 72 hours. The resulting extracts from the leaves and roots were stored at 4°C before further analysis. The percentage yield was calculated using (1).

$$Percentage\ Yield = \frac{m1}{m2} \times 100\% \quad (1)$$

where:

m_1 = mass (g) of the crude extracts

m_2 = mass (g) of the powder

2.2 Total Phenolic Content

The total phenolic content (TPC) of *J. gendarussa* extracts was determined using Folin-Ciocalteu colorimetric method, as in [12] with slight modifications. Briefly, the extracts were prepared in absolute methanol at 1 mg/ml. The extract was mixed with Folin-Ciocalteu reagent, followed by the addition of 20% sodium carbonate solution. The mixture was thoroughly vortexed and then incubated in the dark at room temperature for 30 minutes to allow the color to develop. After incubation, the absorbance of the solution was measured at 760 nm using a Genesys-20 UV-visible spectrophotometer. A standard curve was constructed using gallic acid at 0.025, 0.050, 0.075, 0.100, 0.150, and 0.200 mg/ml concentrations. The phenolic content of each sample was extrapolated from the gallic acid standard curve. The total phenolic content was calculated using formula (2). The TPC values were converted to mmol GAE/g using gallic acid molecular weight of 170.12 g/mol.

$$TPC\ (mg\ GAE/g) = \frac{C \times V}{M} \quad (2)$$

where:

C = TPC concentration in the sample from the calibration curve (mg/mL)

V = volume of extract used (mL)

M = mass of the extract used (g)

2.3 Total Flavonoid Content

The total flavonoid content (TFC) of *J. gendarussa* extracts was determined using aluminum chloride colorimetric method [12]. The extracts were prepared at a concentration of 1 mg/ml.

The extract was mixed with 10% aluminum chloride, distilled water, and 5% sodium nitrate in methanol. The mixture was incubated at room temperature for 30 minutes in the dark. After incubation, the absorbance was measured at 510 nm using a Genesys-20 UV-visible spectrophotometer. A standard curve was prepared using 0.025, 0.050, 0.100, 0.150, and 0.200 mg/ml quercetin. The flavonoid content of each sample was extrapolated from the quercetin standard curve. The total flavonoid content was calculated using formula (3). The TFC values were converted to mmol QE/g using quercetin molecular weight of 302.24 g/mol.

$$TFC\ (mg\ QE/g) = \frac{C \times V}{M} \quad (3)$$

where:

C = TFC level of the sample from quercetin calibration curve (mg/mL)

V = volume of extract used (mL)

M = mass of the extract used (g)

2.4 2,2-diphenyl-1-picrylhyrazyl (DPPH) Radical Scavenging Activity

Free radical scavenging activity of methanolic extracts from the leaf and root of *J. gendarussa* was evaluated using the DPPH assay as previously reported [12].

The samples or ascorbic acid were mixed with 0.25 mM DPPH solution. Methanol was added as a buffer to a final volume of 1 ml. Methanolic crude extracts of *J. gendarussa* leaf and root were assayed at 0.1, 0.3, and 0.5 mg/ml final concentration. 0.1 mg/ml ascorbic acid (A92902, Sigma-Aldrich) served as the positive control. Methanol alone served as the negative control.

0.1, 0.3, and 0.5 mg/ml extracts (diluted in methanol without DPPH solution) were prepared for background absorbance correction for their respective samples. The mixture was vortexed thoroughly to ensure proper mixing and incubated at room temperature for 30 minutes. The mixture was then transferred into a cuvette for absorbance measurement at 517 nm using a Genesys-20 UV-visible spectrophotometer. The absorbance for the samples was corrected with their respective background control. DPPH radical scavenging activity was calculated using (4).

$$Scavenging\ Activity\ (\%) = \left(\frac{A0\text{-}A1}{A0}\right) \times 100\% \quad (4)$$

where:

A0 = absorbance of the negative control

A1 = absorbance of the sample or standard

2.1 Tyrosinase Inhibition Assay

Tyrosinase inhibition activity of *J. gendarussa* extract was evaluated using L-DOPA substrate-based colorimetric assay with some modifications [12]. *J. gendarussa* crude extracts and kojic acid (EvaChem) were assayed at 0.5 mg/ml final concentrations.

Extracts and controls were diluted in 0.05 M potassium phosphate buffer (pH of 6.8). 22.5 U/ml tyrosinase (T3824, Sigma-Aldrich) was added to the samples. Following a 15-minute incubation, 0.5 mM L-DOPA (D9628, Sigma-Aldrich) was added to each sample, and the mixture was further incubated at room temperature for 30 minutes. Kojic acid and methanol served as the positive and negative control, respectively. Due to the inherent color of the extracts, extracts at 0.5 mg/ml (diluted with the buffer without L-DOPA and tyrosinase) were prepared for background absorbance correction for their respective samples. After incubation, the absorbance was measured at 475 nm to determine the degree of dopachrome formation, which reflects tyrosinase activity. After correcting for the background sample absorbance, the percentage of tyrosinase inhibition was calculated using (5).

$$Tyrosinase\ \ Inhibition\ (\%) = \left(\frac{A0 - A1}{A0}\right) \times 100\% \qquad (5)$$

where:

A0 = absorbance of the control (L-DOPA + methanol)

A1 = absorbance of the test sample (L-DOPA + extract or kojic acid)

2.2 Collagenase Inhibition Assay

Collagenase inhibition activity was assessed using a method as in [12]. *J. gendarussa* leaf and root extract were tested at 0.5 mg/ml final concentration. 0.35 mM oleanolic acid and methanol served as the positive and negative controls, respectively.

The extracts and controls were mixed with cold 0.05 M tricine buffer (pH 7.5). 0.1 U/ml collagenase (C0130, Sigma-Aldrich) was added to the mixture and incubated for 15 minutes at room temperature. 0.25 mM FALGPA substrate (F5135, Sigma-Aldrich) was added to all sample mixtures. Extracts at 0.5 mg/ml (diluted with the buffer without FALGPA and enzyme) were prepared for background absorbance correction for their respective samples. Absorbance was measured at 345 nm using a UV-Visible spectrophotometer. After background correction, the percentage of inhibition was calculated using (6).

$$Collagenase\ Inhibition\ (\%) = \left(\frac{1 - B}{A}\right) \times 100\% \qquad (6)$$

where:

A = absorbance of negative control

B = absorbance of the test sample or oleanolic acid

2.3 Statistical Analysis

All data were expressed as the average ± standard deviation (SD) from at least three separate experiments (n=3) except for collagenase assay where n=2. Statistical analyses were performed using GraphPad Prism 5.01. Two-way ANOVA with Bonferroni post-test was used in the DPPH radical scavenging assay. One-way ANOVA with Tukey's multiple comparison was used in tyrosinase and collagenase inhibition assays. Same alphabet indicates a non-significant difference. A p-value of less than 0.05 was considered statistically significant.

3. RESULT AND DISCUSSION

3.1 Extraction Efficacy using Methanol Solvent and Soxhlet Method

Methanolic leaf and root extracts of *J. gendarussa* were obtained using Soxhlet extraction. The percentage yield of the methanolic leaf extract was slightly higher than the root extract (Table 1).

Table 1. Percentage Yield of Methanolic Leaf and Root Extracts of *J. gendarussa*

Extracts	Mass (g) of powder (m_1)	Mass (g) of Crude Extract (m_2)	Percentage of Yield (%)
JGL	20.00	3.88	19.40
JGR	20.00	2.65	13.25

JGL: *J. gendarussa* leaf; JGR: *J. gendarussa* root

The difference in yield may be attributed to copious phytochemical components in different plant parts with varying solubility in methanol. Methanol was chosen in this study based on a previous study that showed significantly higher yield when compared to water [10]. The present study obtained a higher yield than Krishna et al., who reported a 12.72% yield from the leaf using methanol in Soxhlet extraction [10]. Another study also showed methanol as the best solvent to extract phytochemicals from the leaf of *J. gendarussa*, where the yields were 12.3%, 8.2%, and 6.5% for methanol, petroleum ether, and chloroform, respectively [13]. The differences in yield percentage could be due to different extraction conditions, plant materials, and growing conditions. Nonetheless, this study shows that methanol is a suitable solvent for extracting bioactive compounds from *J. gendarussa* with a higher yield from the leaf.

3.2 Total Phenolic and Flavonoid Content J. gendarussa

The total phenolic (TPC) and flavonoid content (TFC) of *J. gendarussa* extracts were determined and are presented in Table 2.

Table 2. TPC and TFC Values of Methanolic Leaf and Root Extracts of *J. gendarussa*

Assay	JGL	JGR	P-value
TPC (mmol GAE/g extract)	0.293 ± 0.018	0.251 ± 0.023	0.068
TFC (mmol QE/g extract)	0.157 ± 0.015	0.172 ± 0.047	0.65

The present study reported higher TPC values compared to TFC values for both extracts, which are in line with the expected results since flavonoids are a subclass of phenolic compounds. The leaves showed a slightly higher TPC value, whereas the root extract showed a higher TFC value, but the differences were not statistically significant. These values were lower than previous studies [10, 14], but better than those reported by Kumaresh & Chowdhury [9].

The slight elevation of TPC in the leaf mirrored a previous report [15] and is likely due to greater light exposure that enhances phenolic compound production through the activation of phenylalanine ammonia-lyase (PAL), a key enzyme in phenolic compound biosynthesis [16]. The slightly higher TFC in the roots is potentially due to their essential roles in plant defence against soil pathogens and, at the same time, mediate symbiotic interactions of the plant with commensal soil microbes [17].

In conclusion, both leaf and root extracts of *J. gendarussa* contain significant amount of phenolic and flavonoid compounds, consistent with previously reported antioxidant activity of the plant.

3.3 DPPH Radical Scavenging Activity

Next, the antioxidant property of the extracts was evaluated using 2,2-diphenyl-1-picrylhdrazyl (DPPH) assay. Both extracts showed dose-dependent radical scavenging activities (Table 3). Two-way ANOVA analysis showed a significant effect of concentration, indicated by the asterisks, but no significant effect of the sample type (plant parts) nor the interaction, indicating similar concentration-response behavior of the extracts in this assay. This result was consistent with the TPC results, where the JGL extract showed higher values in both assays.

Table 3. DPPH Radical Scavenging Activity of JGL, JGR and Ascorbic Acid

Sample	Concentration (mg/ml)	DPPH Scavenging Activity (%)	Conc. effect	Sample effect
JGL	0.1	1.825 ± 2.054	***	n.s
	0.3	12.942 ± 5.455		
	0.5	44.657 ± 2.696		
JGR	0.1	0.796 ± 2.344	***	
	0.3	15.275 ± 5.027		
	0.5	39.771 ± 2.439		
Ascorbic acid	0.1	92.125 ± 1.602		

The assay was repeated four times (n=4). Two-way ANOVA with Bonferroni post test was performed. ***: p-value < 0.001; n.s: not significant

Previous studies showed that methanolic leaf extract of *J. gendarussa* demonstrated antioxidant activities with IC_{50} values ranges from 0.075-0.223 mg/ml [9, 10], which are significantly lower than the present findings. The lower antioxidant activities of these samples are due to the different sample preparation methods. Krishna et al. performed Soxhlet extraction for 24 hours, effectively improve bioactive compound extraction [10]. Kumaresh et al., on the other hand, employed freeze-drying method to preserve the bioactive compounds [9]. The higher antioxidant activity of the JGL compared to JGR could be attributed to the presence of other strong antioxidants in the leaves such as chlorophyll and other carotenoid pigments [18].

Ascorbic acid, as the positive control, exhibited 92.12% radical scavenging activity at 0.1 mg/ml, which was comparable to previous studies that showed 70-84% antioxidant activity at the same concentration [9, 19]. Ascorbic acid was significantly more potent in DPPH scavenging activity than both extracts. The substantial difference between ascorbic acid and the extracts reflects the sample compositions where ascorbic acid sample comprised pure and potent antioxidants, whereas the JGL and JGR extracts encompassed complex mixtures of phytochemicals.

Nevertheless, this study demonstrated that *J. gendarussa* extracts, especially the leaf, contain significant antioxidant compounds.

3.4 Tyrosinase Inhibition Assay

ROS is a key skin aging modulator than can increase the expression and activity of collagenase, tyrosinase, and other enzymes. Thus, antioxidant properties of *J. gendarussa* extracts may limit the activities of these enzymes to slow or prevent skin aging. Thus, the potential inhibition of tyrosinase, a key enzyme in melanin biosynthesis, was assessed by measuring dopachrome formation.

Both leaf and root extract of *J. gendarussa* exhibited moderate tyrosinase inhibitory activity, with a more pronounced effect by the JGL (Figure 1). At 0.5 mg/ml final concentration, JGL showed 47.84 ± 10.16% tyrosinase inhibition, while JGR demonstrated a lower activity at 37.21 ± 12.56%. However, both extracts were significantly lower than kojic acid positive control (91.73 ± 4.24%).

To the best of our knowledge, this is the first study to assess and compare tyrosinase inhibition using *J. gendarussa* leaf and root extracts [6-8]. However, other species of the Justicia genus, such as *Justicia vahlii* and *Justicia secunda*, have been reported to inhibit tyrosinase in vitro [20, 21]. The higher activity of JGL could be attributed to its phytochemical composition. The leaves of *J. gendarussa* were reported to contain numerous phytochemicals such as phytol, garcinone C, L-valine, naringenin, and many others [7, 22]. These compounds have been validated in numerous studies for their tyrosinase inhibition activity [23-25].

The whole *J. gendarussa* plant also contains triterpenes such as lupeol and stigmasterol [26], which have been previously shown to inhibit tyrosinase [27, 28]. Specifically, stigmasterol inhibits tyrosinase by neutralizing ROS and blocking alpha-melanin-stimulating hormone activity [28]. Thus, this study demonstrates a novel property of *J. gendarussa* extracts, particularly the leaf, as a potential natural tyrosinase inhibitor.

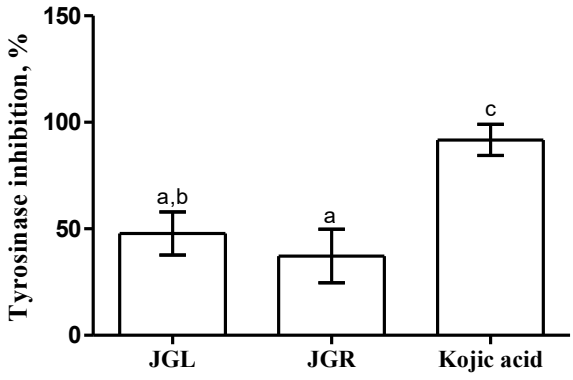

Fig. 1. Tyrosinase Inhibition Activity of JGL and JGR.

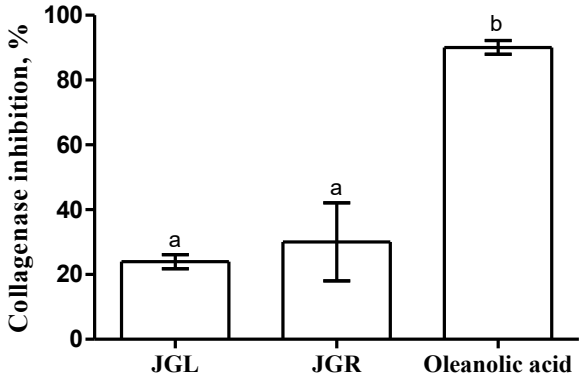

Fig. 2. Collagenase Inhibition Activity of JGL and JGR.

3.5 Collagenase Inhibition Activity

Collagenase plays a critical role in skin aging by degrading collagen in the dermis. This enzymatic degradation contributes to increased skin laxity, wrinkle formation, and sagging [2]. ROS has been shown to modulate the expression and activity of collagenase [3]. In this final analysis, the methanolic JGL and JGR extracts were evaluated for their collagenase inhibition potential (Figure 2).

0.5 mg/ml JGR extract displayed slightly higher collagenase inhibition (30.05 ± 12.04%) compared to JGL extract (23.97 ± 2.20%), but the overall inhibitory effects were relatively low compared to 0.35 mM oleanolic acid positive control (81.47 ± 2.15%).

A previous study revealed significant collagenase inhibition with IC_{50} values ranging from 0.0042.75 to 1564.41 µg/ml for methanolic *J. gendarussa* leaf extraction and toluene, chloroform, ethyl-acetate, and butanol fractions [11]. However, these values are not directly comparable to the present study because a different extraction method and a different collagenase assay were used to assess collagenase inhibition.

Some phytochemicals in *J. gendarussa* leaves and roots that may contribute to this activity include garcinone C, kaempferol, and L-valine [29-31]. The root of *J. gendarussa* contains apigenin [7], well-known for its collagenase inhibition during inflammation [32]. Further studies are required to identify other phytochemicals that contribute to this activity.

In summary, although the extracts did not demonstrate potent tyrosinase and collagenase inhibition, their bioactive phytochemical composition suggests potential for future formulation into antiaging products.

It is important to note that these findings are only based on in vitro biochemical assays, which lack direct clinical translation. This limitation warrants further studies to identify specific phytochemicals in *J. gendarussa* that contribute to the antiaging properties. Employing other antiaging assays and advanced pre-clinical models are also essential to properly establish the antioxidant and antiaging properties of *J. gendarussa*.

4. CONCLUSION

This study demonstrated that the methanolic extracts of *J. gendarussa* leaves and roots possess antioxidant and antiaging properties. Both extracts contain varied levels of phenolic and flavonoid compounds. The extracts showed dose-dependent DPPH radical scavenging activities. Tyrosinase inhibition was higher in the leaf extract, suggesting potential for skin whitening application while collagenase inhibition was generally low in both extracts. These findings suggest that *J. gendarussa* may serve as a promising local plant in developing novel natural-based skincare products.

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