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Anti-inflammatory, antioxidant activity, and acetylcholinesterase inhibitory activity of leaf extract of *Juglans regia:* Insight into the treatment of neurodegenerative diseases

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Abstract

Neuroinflammation, depletion of acetylcholine and neuronal oxidative stress are hallmarks of neurodegenerative diseases such as Alzheimer's disease. This study sought to investigate *in vitro* acetylcholinesterase inhibitory activity, free radical scavenging, antioxidant activity, and anti-inflammatory activity of aqueous leaf extract of *Juglans regia*. The antioxidant, anti-inflammatory, and acetylcholinesterase-inhibitory activities of the extract were evaluated using different appropriate techniques. The extract showed substantial free radical scavenging and antioxidant activity by markedly scavenging hydroxyl radical, DPPH, nitric oxide, hydrogen peroxide, with concomitant high free radical reducing power. Similarly, lipid peroxidation and inflammation were significantly inhibited by the extract. The extract elicited acetylcholinesterase-inhibitory potential of 45.49%, suggesting they could be beneficial in managing neurodegenerative diseases. These biological activities can be attributed to the phytochemical constituents of the extract. Preliminary phytochemical screening of the extract showed the presence of polyphenols, flavonoids, and tannins and GCMS identified phytochemicals including vanillic acid, p-Coumaric acid, caffeic acid, squalene. This study concluded that the aqueous leaf extract of *J. regia* exhibited significant antioxidant, anti-inflammatory and acetylcholinesterase-inhibitory activities which could be beneficial in mitigating the pathological features of Alzheimer's disease and other neurodegenerative diseases, thus, it could be explored as health-promoting dietary supplements/ products.

Keywords: acetylcholinesterase; anti-inflammatory; antioxidant; neurodegenerative diseases; phytochemicals; walnut

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Introduction

The therapeutic benefits of medicinal plants have been employed extensively throughout history to treat a variety of human ailments, including neurodegenerative diseases (Oladele *et al.,* 2020, 2020a, 2021). Their natural ingredients and phytochemical components, which have promising impacts on human health, are the source of their therapeutic relevance (Bello *et al.,* 2015; Oyewole *et al.,* 2017; Oyeleke *et al.,* 2021; Oladiji and Oladele, 2023). Some of these natural substances possess antioxidant, anti-inflammatory, anti-apoptotic, and modulatory properties that influence cellular processes to provide health-beneficial effects (Bello *et al.,* 2015; Oyewole *et al.,* 2017; Oyeleke *et al.,* 2021; Oladiji and Oladele, 2023).

Alzheimer's disease (AD) is one of the neurodegenerative disorders and the most common cause of dementia in adult individuals with global prevalence of 24 million and estimated to increase four times in 2050 (Balkis *et al.,* 2015; ADI, 2019; Kumar *et al.,* 2022). Although the exact pathogenic process of neurodegenerative disease is not fully unknown, the "cholinergic hypothesis" postulates that it involves a reduction in the neurotransmitter acetylcholine in the brain. The medications currently prescribed for AD patients boost the brain's acetylcholine levels, which are essential for central cholinergic transmission (Wszelaki *et al.,* 2010). Acetylcholine is degraded by the enzyme acetylcholinesterase to choline and an acetyl group after being transported across neural synapses (Balkis *et al.,* 2015). Because of this, acetylcholine is not hydrolyzed when acetylcholinesterase inhibitors are used, retaining its function as a neurotransmitter (Knapp *et al.,* 1994; Voet and Voet, 1995). The search is still ongoing for new medications that are more effective at penetrating the brain, less harmful, and with a high bioavailability (Knapp *et al.,* 1994). However, several of the medications authorized for the treatment of AD symptoms have been linked to hepatotoxicity (Knapp *et al.,* 1994), a higher risk of urine incontinence (Gill *et al.,* 2005), a higher risk of bradycardia and other serious cardiovascular consequences (Howes, 2014), pulmonary issues (Helou and Rhalimi, 2010), and involuntary weight loss (Tamura *et al.,* 2007).

A target for the development of mechanism-based inhibitors, the acetylcholinesterase enzyme (AChE) is involved in the breakdown of the neurotransmitter acetylcholine. The most effective medications for treating cognitive symptoms of AD at the moment are AChE inhibitors like galantamine, donepezil (Guo *et al.,* 2010), or rivastigmine (Khan *et al.,* 2009). These medications also have other potential therapeutic uses for treating other neurodegenerative diseases. Studies on the treatment and/or prevention of Alzheimer's disease have concentrated on naturally occurring acetylcholinesterase (AChE) inhibitors from plants, particularly polyphenolic compounds, such as flavonoids, with inhibitory potencies comparable to those of the currently recommended AChE inhibitor medications (Heinrich *et al.,* 2004; Katalinic *et al.,* 2010; Kim *et al.,* 2011; Hogan, 2014), of which the alkaloid galantamine is a prime instance (Guo *et al.,* 2010). The fact that this substance is frequently present in foods gives it the benefit of being more readily tolerated and less expensive, and its antioxidant activity and potent metal chelator properties may also help to lessen the oxidative stress that damages the tissues linked to Alzheimer's disease (Balkis *et al.,* 2015).

The biological activity of walnut (*Juglans regia*) leaves was examined in this study. *J. regia*, also referred to as the walnut tree, is a member of the Juglandaceae family and is a fragrant transitory tree that grows in profusion in Kashmir's North-Western Himalayas, where 88% of the world's walnut supply is produced (Acarsoy-Bilgin, 2022). Numerous significant phytochemicals have been taken advantage of as a result of intensive research into the tree's phytochemistry (Bhat *et al.,* 2023). Walnut leaves contain a variety of bioactive substances, such as tannins, flavonoids, and phenolic compounds. These substances have anti-inflammatory, anti-microbial, antioxidant, and anti-cancer properties. They can be used in conventional medicine to treat ailments like diabetes, cardiovascular disease, some types of cancer, and skin disorders (Bhat *et al.,* 2023). Similarly, a wide range of illnesses, including infectious diseases, cancer, skin disorders, scrofula, eczema,

asthma, arthritis, stomach aches, sinusitis, diarrhoea, helminthiasis, and various endocrine diseases like thyroid dysfunctions, anorexia, and diabetes mellitus, have been treated with a great deal of success using the medicinal plant *J. regia* Linn. (Hayes *et al.,* 2016; Gupta *et al.,* 2019).

This study was designed to elucidate *in vitro* free radical scavenging activity, antioxidant, lipid peroxidation inhibitory, anti-inflammatory, and *in vitro* acetylcholinesterase inhibitory activities of aqueous leaf extract of *J. regia*.

Materials and Methods

Sample collection and preparation

The newly collected leaves of *J. regia* for this investigation were from a farm in the town of Iree in the state of Osun. The sample was air dried and processed into a fine powder using a grinding mill. For additional analysis, the powdered sample was stored in an airtight container. The powdered sample was weighed and immersed in 6 volumes of distilled water with constant stirring for 72 hours. Filtrate was used to obtain the extract, and the paste was then freeze-dried.

Phytochemical and Gas Chromatography - Mass Spectroscopy (GC-MS) analysis of the extract

Qualitative screening and of the aqueous leaf extract of *J. regia* were carried out using appropriate methods as previously documented (Oyeleke *et al.,* 2021). The phytochemicals present in the aqueous leaf extract of *J. regia* were determined using GC-MS (Model: QP2010 plus Shimadzu, Japan) consisting of an AOC-20i auto-sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS). The mass spectrum was interpreted using the National Institute of Standards and Technology (NIST) database, which contains over 62,000 patterns. The unknown components' fragmentation pattern spectra were compared to those of known components stored in the NIST library (NIST 11). Each phytocomponent's relative percentage was calculated by comparing its average peak area to the total area. The name, molecular weight, and structure of the components of the test materials were determined (Owokotomo *et al.,* 2015; Oladele *et al.,* 2021).

Determination of in vitro antioxidant activity

Ferric reducing power assay was carried out using an assay method described by Shahi *et al*. (2020). The assay procedure of Oyeleke *et al*. (2021) was employed to determine the DPPH (1,1-diphenyl-1,2-picryl hydrazyl) – radical scavenging activity of the extract. The hydroxyl radical scavenging activity of extract was carried out employing the assay protocol of Kim and Minamikawa (1997) as described by Shahi *et al*. (2020). The ability of the extract to scavenge hydrogen peroxide (H_2O_2) was determined according to Nabavi *et al*. (2008, 2009). Nitric oxide radical scavenging activity of the extract was determined as described by Ebrahimzadeh *et al*. (2009). The lipid peroxidation inhibition assay (LPI) was performed using a slightly modified method by Liu *et al*. (2013). Catalase activity was measured using the procedure outlined by Cohen e*t al*. (1970). ABTS (2, 2-a zino- bis(3- ethylbenzothiazoline – 6- sulfonic acid) radical scavenging activity was determined using method of Re *et al*. (1999).

Determination of In vitro anti-inflammatory activity

The modified method developed by Oyedapo *et al*. (2010) was used to determine the red blood cell membrane stability potential of the extract. The lipoxygenase inhibitory activity of the extract was determined used appropriate protocol (Avelia *et al*., 2017).

Acetylcholinesterase inhibitory assay

Acetylcholinesterase inhibitory activity of the extract was investigated employing the Ellman et al. (1961) method with slight modifications. Aliquots of rat brain homogenate was used to measure acetylcholinesterase activity, a marker for cholinergic neurotransmission. Typically, the acetylcholinesterase inhibitory activity of the protein fractions in the brain homogenate was measured by adding aliquot (2.6 mL) of phosphate buffer (0.1 M, pH 7.4), 0.8 mL of the respective protein fractions, 0.1 mL of 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), and 0.4 mL of the homogenate. The reaction mixture was then spiked with 0.1 mL of acetylthiocholine iodide solution. The absorbance was measured at 412 nm, and the change in absorbance was recorded every two minutes for ten minutes. The rise in colour produced when thiocholine reacts with DTNB was used to determine the rate of acetylcholinesterase activity. The rate of acetylcholinesterase activity was computed and represented as moles/min/g tissue.

$$Rate = \frac{\Delta\, Absorbance}{min}$$

Enzyme activity = %Rate

Statistical analysis

Data obtained during the experiment were presented as mean ± standard error, and subjected to Student's t-test for comparison between two sets of data. Differences were considered significant at $p<0.05$.

Results

In vitro free radical and antioxidant activity of leaf extract of J. regia

Tables 1-6 showed the results of the *In vitro* free radical and antioxidant activity of leaf extract of *J. regia*. The DPPH radical scavenging activity of the extract was shown in Table 1. The result revealed that leaf extract of *J. regia* scavenged DPPH radical in a concentration dependent pattern from 11.38 to 51.16%. Also, in all the tested concentrations, leaf extract of *J. regia* demonstrated higher scavenging activity than ascorbic acid. Table 2 showed the ABTS radical scavenging activity of the leaf extract of *J. regia* and the standard antioxidants (gallic acid and ascorbic acid) used in this study. The scavenging activity is concentration dependent and at 800 µg/ml, the extract with 45.41% inhibition showed higher inhibitory effect than ascorbic acid (40.35%), indicating that the extract could serve as better antioxidant agent. The result of *in vitro* hydrogen peroxide inhibitory activity of the leaf extract of *J. regia* and the standard antioxidants was displayed in Table 3. Gallic acid showed the highest inhibitory (61.78 ± 2.51% at 800 µg/ml, while the extract and ascorbic acid has 47.30 ± 0.64% and 53.53 ± 2.55% respectively. At 50 µg/ml, leaf extract of *J. regia* demonstrated the highest hydrogen peroxide inhibitory activity of 10.26 ± 1.36% when compared with gallic acid (10.10 ± 1.01%) and ascorbic acid (8.08 ± 0.38%). In Table 4, both the leaf extract of *J. regia* and the standard antioxidants (gallic acid and ascorbic acid) showed substantial *in vitro* scavenging activity against nitric oxide radical. The extract and the standards scavenge nitric oxide radical in a concentration dependent pattern. Although, in all the concentration tested used in this study, the standards have higher activity than the extract, the activity is similar, suggesting that the nitric oxide scavenging activity of the standards and extract is similar.

Table 1. DPPH radical scavenging activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Gallic acid	Ascorbic acid
50	11.38 ± 0.62[a]	15.94 ± 2.75 [a]	9.36 ± 1.17 [a]
100	19.03 ± 1.04 [b]	27.17 ± 4.81 [b]	16.42 ± 3.32 [b]
200	27.94 ± 2.35 [c]	38.01 ± 3.25 [c]	25.08 ± 1.76 [c]
400	39.28 ± 1.06 [d]	52.22 ± 0.93 [d]	35.67 ± 1.80 [d]
800	51.16 ± 1.77 [e]	63.84 ± 2.44 [e]	46.22 ± 1.41 [e]

Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different (P < 0.05). ALEJR: aqueous leaf extract of *J. regia*.

Table 2. ABTS radical scavenging activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Gallic acid	Ascorbic acid
50	7.21 ± 1.25 [a]	10.11 ± 2.39 [a]	8.81 ± 0.54 [a]
100	13.07 ± 1.39 [b]	21.95 ± 2.03 [b]	15.39 ± 0.86 [b]
200	20.95 ± 1.54 [c]	32.73 ± 1.40 [c]	25.48 ± 1.21 [c]
400	31.89 ± 1.11 [d]	40.01 ± 1.81 [d]	37.77 ± 2.22 [d]
800	45.41 ± 0.53 [e]	55.27 ± 1.90 [e]	40.35 ± 4.04 [e]

Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different (P < 0.05). ALEJR: aqueous leaf extract of *J. regia*.

Table 3. Hydrogen peroxide scavenging activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Gallic acid	Ascorbic acid
50	10.26 ± 1.36 [a]	10.10 ± 1.01 [a]	8.08 ± 0.38 [a]
100	13.29 ± 1.16 [b]	15.48 ± 1.82 [b]	16.66 ± 1.27 [b]
200	16.16 ± 1.52 [c]	24.74 ± 1.67 [c]	24.07 ± 1.40 [c]
400	26.26 ± 2.63 [d]	33.67 ± 1.03 [d]	30.47 ± 1.38 [d]
800	47.30 ± 0.64 [e]	61.78 ± 2.51 [e]	53.53 ± 2.55 [e]

Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different (P < 0.05). ALEJR: aqueous leaf extract of *J. regia*.

Table 4. Nitric oxide radical scavenging activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Gallic acid	Ascorbic acid
50	8.62 ± 1.71 [a]	18.11 ± 2.80 [a]	10.85 ± 1.41 [a]
100	12.67 ± 0.24 [b]	23.35 ± 1.94 [b]	11.18 ± 1.48 [b]
200	18.11 ± 2.04 [c]	36.27 ± 1.73 [c]	13.66 ± 0.75 [c]
400	34.12 ± 1.86 [d]	42.31 ± 2.42 [d]	29.59 ± 1.42 [d]
800	45.71 ± 2.05 [e]	49.48 ± 1.41 [e]	45.01 ± 0.73 [e]

Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different (P < 0.05). ALEJR: aqueous leaf extract of *J. regia*.

The lipid peroxidation inhibitory activity of the extract was showed in Table 5. The result revealed that leaf extract of *J. regia* inhibited lipid peroxidation in a concentration dependent pattern. Moreso, in all the tested concentrations, leaf extract of *J. regia* demonstrated higher lipid peroxidation inhibitory activity than ascorbic acid and gallic acid. The result of *in vitro* catalase antioxidant activity of the leaf extract of *J. regia* and the standard antioxidants was presented in Table 6. At 800 µg/ml, leaf extract of *J. regia* demonstrated the highest antioxidant activity of 299.75 ± 4.06 unit/mg protein, while gallic acid and ascorbic acid showed antioxidant activity of 293.15 ± 6.93 unit/mg protein and 298.52 ± 2.57 unit/mg protein respectively.

Table 5. Lipid peroxidation inhibitory activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Gallic acid	Ascorbic acid
50	11.41 ± 1.54 [a]	13.68 ± 1.79 [a]	16.24 ± 1.13 [a]
100	20.49 ± 1.65 [b]	31.63 ± 1.21 [b]	30.92 ± 1.99 [b]
200	30.70 ± 1.22 [c]	40.42 ± 2.53 [c]	40.28 ± 3.69 [c]
400	42.34 ± 0.43 [d]	48.65 ± 2.48 [d]	48.08 ± 2.11 [d]
800	57.30 ± 1.55 [e]	53.04 ± 1.65 [e]	56.02 ± 2.37 [e]

Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different (P < 0.05). ALEJR: aqueous leaf extract of *J. regia.*

Table 6. Catalase activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Gallic acid	Ascorbic acid
50	262.30 ± 3.33 [a]	257.24 ± 4.59 [a]	262.32 ± 1.63 [a]
100	268. 77 ± 2.99 [b]	262.75 ± 3.73 [a]	267.80 ± 2.05 [b]
200	281.43 ± 5.26 [c]	274.39 ± 4.26 [b]	280.63 ± 1.41 [c]
400	298.49 ± 4.51 [d]	292.06 ± 5.07 [c]	295.39 ± 2.52 [d]
800	299.75 ± 4.06 [d]	293.15 ± 6.93 [c]	298.52 ± 2.57 [d]

Catalase (*Unit/mg protein)*. Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different (P < 0.05). ALEJR: aqueous leaf extract of *J. regia.*

In vitro anti-inflammatory activity of leaf extract of J. regia

Membrane stability test and lipoxygenase inhibition assay were used to determine the *in vitro* anti-inflammatory activity of leaf extract of *J. regia* and ibuprofen was used as standard. The results of these assays were presented in Tables 7 and 8. The leaf extract of *J. regia* demonstrated substantial membrane stability potential and lipoxygenase inhibitory activity in a concentration dependent pattern. At 800 µg/ml, leaf extract of *J. regia* displayed 58.98 ± 3.05% while ibuprofen displayed 76.27 ± 4.19% membrane stability activity (Table 7). At 50 µg/ml, leaf extract of *J. regia* (20.98 ± 3.61%) demonstrated higher lipoxygenase inhibitory activity than ibuprofen (20.87 ± 4.32%). At 100 µg/ml, 200 µg/ml and 400 µg/ml, both the extract and standard anti-inflammatory drug (ibuprofen) showed similar anti-inflammatory activity. However, at 800 µg/ml, ibuprofen displayed higher anti-inflammatory activity than the extract (Table 8).

Table 7. Membrane stability activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Ibuprofen
50	12.82 ± 1.51 [a]	13.55 ± 2.32 [a]
100	27.11 ± 2.64 [b]	28.81 ± 3.56 [b]
200	35.42 ± 2.73 [c]	50.84 ± 2.46 [c]
400	40.67 ± 2.97 [d]	61.01 ± 3.95 [d]
800	58.98 ± 3.05 [e]	76.27 ± 4.19 [e]

Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different (P < 0.05). ALEJR: aqueous leaf extract of *J. regia.*

Table 8. Lipoxygenase inhibitory activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Ibuprofen
50	20.98 ± 3.61 [a]	20.87 ± 4.32 [a]
100	32.02 ± 1.86 [b]	32.02 ± 1.86 [b]
200	43.27 ± 2.69 [c]	43.06 ± 2.11 [c]
400	52.13 ± 1.15 [d]	51.91 ± 2.57 [d]
800	69.11 ± 4.75 [e]	73.11 ± 4.75 [e]

Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different (P < 0.05). ALEJR: aqueous leaf extract of *J. regia.*

In vitro acetylcholinesterase inhibitory activity of leaf extract of J. regia

The acetylcholinesterase inhibitory activity of the extract was showed in Table 9. The result revealed that leaf extract of *J. regia* inhibited acetylcholinesterase activity in a concentration dependent pattern. The percentage acetylcholinesterase inhibitory activity of leaf extract of *J. regia* at 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml are 13.03 ± 2.22%, 27.64 ± 2.70%,32.54 ± 1.92%, 40.19 ± 1.08%, and 45.49 ± 1.02% respectively, while percentage acetylcholinesterase inhibitory activity of standard at the same concentrations are 21.66 ± 2.67%, 31.37 ± 2.55%, 46.96 ± 1.78%, 53.43 ± 1.37% and 57.94 ± 1.18%.

Table 9. Acetylcholinesterase inhibitory activity of aqueous leaf extract of *J. regia*

Concentration (µg/ml)	ALEJR	Galantamine
50	13.03 ± 2.22 [a]	21.66 ± 2.67 [a]
100	27.64 ± 2.70 [b]	31.37 ± 2.55 [b]
200	32.54 ± 1.92 [c]	46.96 ± 1.78 [c]
400	40.19 ± 1.08 [d]	53.43 ± 1.37 [d]
800	45.49 ± 1.02 [e]	57.94 ± 1.18 [e]

Values are expressed as mean ± standard deviation. Values followed by different letters are significantly different ($P < 0.05$). ALEJR: aqueous leaf extract of *J. regia*.

Phytochemical constituent of leaf extract of J. regia

The preliminary phytochemical screening of the leaf extract of *J. regia* to rich in alkaloids, flavonoids, glycosides, saponin, tannin, phenol, and coumarins. Figure 1 showed the GCMS spectrum of phytochemical profiling of leaf extract of *J. regia.* Table 10 showed the 23 identified phytochemicals present in the leaf extract of *J. regia* which include benzaldehyde, 4-hydroxy-, 1,3,5-benzenetriol, salicylic acid, benzeneethanol, 4-hydroxy-, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, benzeneacetic acid, 4-hydroxy-, vanillic acid, benzoic acid, 3,4-dihydroxy-, myo-inositol, 2-propenoic acid, 3-(3-hydroxyphenyl)-, p-Coumaric acid, caffeic acid, fructose, glucose, methyl stearate, squalene. The structure of these identified phytochemicals is presented in Figure 2.

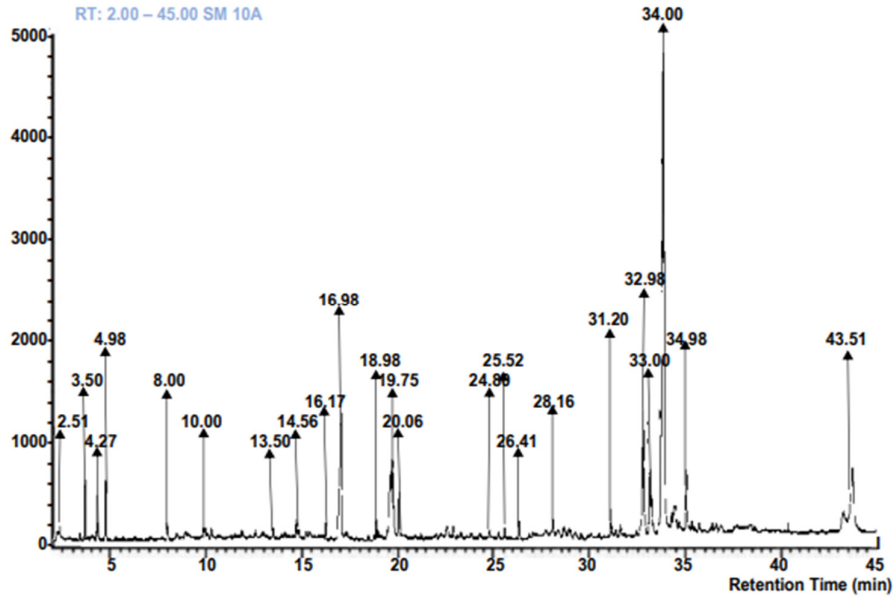
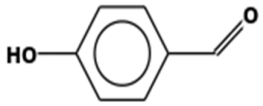
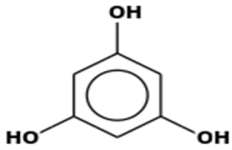
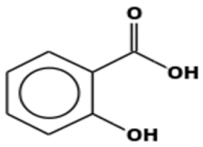
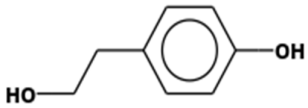
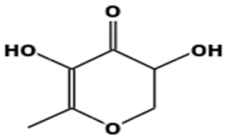
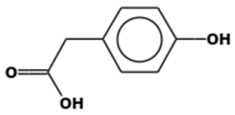
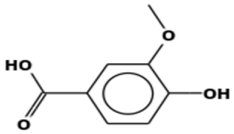
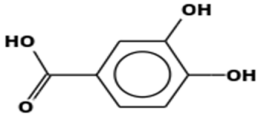


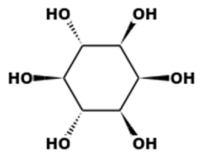
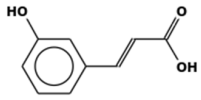
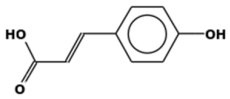
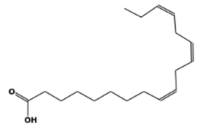
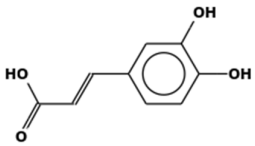
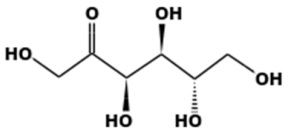
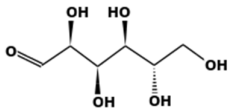
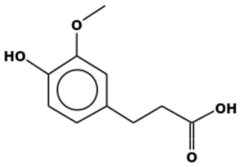
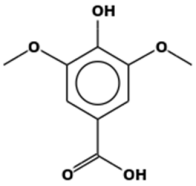
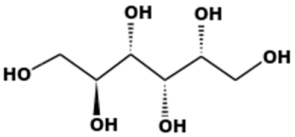
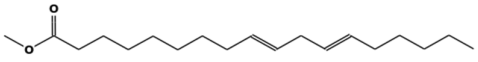
Figure 1. GC-MS spectrum of leaf extract of *J. regia*

Table 10. GC-MS identified phytochemicals from leaf extract of *J. regia*

S/N	RT	Name of Compound identified	Mol. Formula	MW	Peak Area (%)	Comp (wt%)	m/z
1	2.51	Benzaldehyde, 4-hydroxy-	$C_7H_6O_2$	122	1.32	1.68	65, 93, 122
2	3.50	1,3,5-Benzenetriol	$C_6H_6O_3$	126	4.49	5.13	52, 69, 126
3	4.27	Salicylic acid	$C_7H_6O_3$	138	3.32	3.00	64, 92, 138
4	4.98	Benzeneethanol, 4-hydroxy-	$C_8H_{10}O_2$	138	3.33	4.07	77, 107, 138
5	8.00	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	1.34	1.78	43, 101, 144
6	10.00	Benzeneacetic acid, 4-hydroxy-	$C_8H_8O_3$	152	1.35	1.90	77, 107, 152
7	13.50	Vanillic acid	$C_8H_8O_4$	168	1.36	1.96	97, 153, 168
8	14.56	Benzoic acid, 3,4-dihydroxy-	$C_7H_6O_4$	154	1.40	2.00	45, 73, 154
9	16.17	Myo-Inositol	$C_6H_{12}O_6$	180	1.39	0.57	60, 73, 180
10	16.98	2-Propenoic acid, 3-(3-hydroxyphenyl)-	$C_9H_8O_3$	164	9.28	10.18	65, 91, 164
11	18.95	p-Coumaric acid	$C_9H_8O_3$	164	1.35	2.36	65, 119, 164
12	19.75	9,12,15-Octadecatrienoic acid (Z,Z,Z)-	$C_{18}H_{30}O_2$	278	6.34	7.15	43, 79, 278
13	20.06	Caffeic acid	$C_9H_8O_4$	180	3.38	3.28	77, 134, 180
14	24.80	Fructose	$C_6H_{12}O_6$	180	0.66	0.43	43, 73, 180
15	25.52	Glucose	$C_6H_{12}O_6$	180	0.65	0.21	43, 60, 180
16	26.41	β-(4-Hydroxy-3-methoxyphenyl)propionic acid	$C_{10}H_{12}O_4$	196	0.64	1.10	77, 133, 196
17	28.16	Benzoic acid, 4-hydroxy-3,5-dimethoxy-	$C_9H_{10}O_5$	198	1.69	2.26	109, 127, 198
18	31.20	Sorbitol	$C_6H_{14}O_6$	182	1.60	0.31	43, 73, 182
19	32.98	Methyl stearate	$C_{19}H_{38}O_2$	294	9.29	10.24	41, 74, 294
20	33.00	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	4.04	3.90	43, 73, 228

21	34.00	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	32.93	26.31	43, 73, 256
22	34.98	1,2-Benzenedicarboxylic acid, diheptyl ester	$C_{22}H_{34}O_4$	362	4.14	5.00	41, 149, 362
23	43.51	Squalene	$C_{30}H_{50}$	410	4.68	5.08	41, 69, 410

Name of Compound identified	Structures
Benzaldehyde, 4-hydroxy-	
1,3,5-Benzenetriol	
Salicylic acid	
Benzeneethanol, 4-hydroxy-	
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	
Benzeneacetic acid, 4-hydroxy-	
Vanillic acid	
Benzoic acid, 3,4-dihydroxy-	

Myo-Inositol	
2-Propenoic acid, 3-(3-hydroxyphenyl)-	
p-Coumaric acid	
9,12,15-Octadecatrienoic acid (Z,Z,Z)-	
Caffeic acid	
Fructose	
Glucose	
β-(4-Hydroxy-3-methoxyphenyl)propionic acid	
Benzoic acid, 4-hydroxy-3,5-dimethoxy-	
Sorbitol	
Methyl stearate	

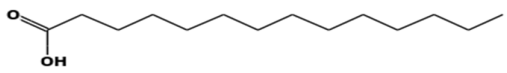
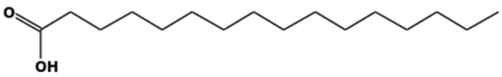
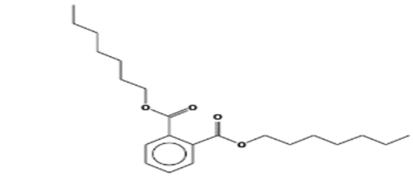
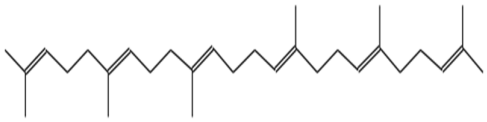
Tetradecanoic acid	
n-Hexadecanoic acid	
1,2-Benzenedicarboxylic acid, diheptyl ester	
Squalene	

Figure 2. Structures of GC-MS identified phytochemicals from leaf extract of *J. regia*

Discussion

Recent developments in several oxidant-related diseases have made the predominance of oxidants clear. Major chronic diseases like neurodegenerative disease, diabetes, asthma, cancer, arteriosclerosis, and inflammation, may all be prevented or treated with the use of antioxidant-rich pharmaceutical compounds (Reuter *et al.,* 2013; Oladele *et al.,* 2019). This study examined the anti-inflammatory, antioxidant activity, and acetylcholinesterase inhibitory activity of leaf extract of *J. regia.*

An essential characteristic of an antioxidant is proton-radical scavenging activity, which may be assessed using the DPPH radical scavenging assay. A protonated free radical called DPPH creates a violet solution in ethanol and has the property of having an absorbance maximum at 517 nm. It is stable at room temperature. The DPPH is decreased when an antioxidant ingredient is present, and a yellow ethanol solution is the result. According to Deodhar *et al*. (2007), it is well known that blocking one or more phenol groups in flavonoids and their derivatives, such as chalcones, decreased in some cases significantly the antioxidant abilities. The findings of this research demonstrated the antioxidant capacity of leaf extract of *J. regia*. Table 1 showed the antiradical characteristics of leaf extract of *J. regia*. As the outcome demonstrates, the extract is more effective at scavenging DPPH than ascorbic acid, a common antioxidant. The extract's high concentration of phenolic chemicals as shown in the GCMS analysis result may be the cause of its comparatively high activity.

The decrease of the radical cation at 734 nm is used by the ABTS technique to evaluate the antioxidant activity of leaf extract of *J. regia*. According to Chakraborty and Paulraj (2010), the decolorization of the $ABTS^{+}$ cation demonstrates an antioxidant's ability to give hydrogen atoms or electrons to render this radical species inactive (Chakraborty and Paulraj, 2010). The leaf extract of *J. regia* considerably outperformed ascorbic acid at scavenging the ABTS radical in this study. This finding suggests that the leaf extract of *J. regia* may be a more effective natural source of antioxidants than ascorbic acid due to its increased antioxidant activity.

The leaf extract of *J. regia* also displayed concentration-dependent hydrogen peroxide breakdown activity, as indicated in Table 3. A weak oxidizing agent, hydrogen peroxide can directly inactivate a small number of enzymes, mainly by oxidizing crucial thiol (-SH) groups. Upon entering the cell, hydrogen peroxide can likely react with Fe^{2+} and potentially Cu^{2+} ions to generate a hydroxyl radical, which may be the cause of

many of its hazardous effects (Halliwell, 1993). Hydrogen peroxide can quickly permeate cell membranes. Therefore, it is advantageous for cells from a biological perspective to regulate the amount of hydrogen peroxide that is permitted to build up. The ability of leaf extract of *J. regia* to combat free radicals and operate as an antioxidant may be at least partially responsible for the ability of the extract to decompose H_2O_2.

The amounts of malondialdehyde, a lipid peroxidation product, are used to measure the importance of lipid peroxidation in free radical disease (Cheeseman and Holley, 1993). By reducing membrane fluidity and changing the activity of membrane-bound enzymes and receptors, increased lipid peroxidation reduces membrane activity. The byproducts of lipid peroxidation harm the majority of the body's cells and are linked to a number of illnesses, including atherosclerosis and brain impairment (Borek, 2001; Oladele *et al.,* 2020). The ability of leaf extract of *J. regia* to prevent lipid peroxidation was evaluated and compared to standards. In comparison to the two standards utilized in this experiment (ascorbic and garlic acids), the leaf extract of *J. regia* showed the maximum lipid peroxidation inhibitory potency. This suggested that the leaf extract of *J. regia* was even more effective than the standard medication.

In this study, three assays were employed to investigate the anti-inflammatory potency of leaf extract of *J. regia* which include nitric oxide, membrane stability, lipoxygenase assays. Numerous physiological and pathologic events are caused by nitric oxide (NO), a free radical and biomarker for inflammation that has the potential to be harmful (Marcocci *et al.,* 1994; Oladele *et al.,* 2020). The leaf extract of *J. regia* that was employed in this study scavenged NO radical in a concentration dependent pattern. This further confirm the antioxidant and anti-inflammatory potency of leaf extract of *J. regia.*

At various concentrations, the capacity of leaf extract of *J. regia* to maintain the membrane of the red blood cell was examined. The outcomes showed that the extract had large percentage activity that affected membrane stability. These effects were comparable to ibuprofen (a non-steroidal anti-inflammatory medication), which had the highest percentage membrane activity.

One important enzyme, lipoxygenase, has been linked to numerous inflammation-mediated diseases, including cancer, arthrosclerosis, hypertension, and diabetes (Osher *et al.,* 2006). It may be possible to develop novel compounds with anti-inflammatory properties by inhibiting the activity of this enzyme. The lipoxygenase inhibition activity in this investigation is proportional to the extract concentration utilized for the test. The lipoxygenase inhibition activity increases with sample concentration, which indicates that inhibition will reduce the amount of hydroperoxyoctadieneoic acid produced as a result of the reaction between the substrate and lipoxygenase. The extract exhibits considerable lipoxygenase inhibitory activity according to this test, using ibuprofen as a positive control.

A number of neurological illnesses, including myasthenia gravis, senile dementia, ataxia, and Alzheimer's disease, are thought to be treatable with the inhibition of acetylcholinesterase (AChE), the major enzyme in the breakdown of acetychloline (Oladele *et al.,* 2020b; 2021b). According to Howes and Houghton (2003), plants have historically been utilised to improve cognitive performance and treat various symptoms of Alzheimer's disease. A version of the approach outlined in the literature was used to determine the AChE inhibition. By comparing the sample to the standard, it was discovered that it was active against acetylcholinesterase.

Conclusions

Based on the findings of this study, leaf extract of *J. regia* has substantial anti-inflammatory, antioxidant activity, and acetylcholinesterase inhibitory activity. These reported activities leaf extract of *J. regia* is essential to mitigate the cardinal pathological hallmarks of Alzheimer's diseases which include neuronal oxidative stress, neuroinflammation, neuronal cell loss and depletion of neurotransmitter, acetylcholine. Similarly, leaf extract of *J. regia* is abundant in antioxidants and phytochemicals. Consumption of leaf extract of *J. regia* may enhance

brain functions and lower the risk of oxidant-mediated diseases such as neurodegenerative diseases, cancer and heart disease.

Authors' Contributions

ORA: conceptualization, investigation, writing. BMA: investigation, laboratory analysis. JOO: conceptualization, investigation, data analysis, resources, writing. FA: investigation, laboratory analysis. OOE: investigation, laboratory analysis. SAS: investigation, laboratory analysis. OAO: investigation, laboratory analysis. AAA: investigation, laboratory analysis. OAA: investigation, laboratory analysis. LAA: investigation, laboratory analysis. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Acarsoy-Bilgin N (2022). Morphological characterization of pollen in some varieties of walnut (*Juglans regia*). International Journal of Fruit Science 22:471-480. *https://doi.org/10.1080/15538362.2022.2060895*

Alzheimer's Disease International (ADI), World Alzheimer Report (2019). Attitudes to Dementia, Alzheimer's Disease International, London, UK, 2019.

Avelia S, Mauldina MG, Elya B (2017). Antioxidant activity and lipoxygenase inhibitory assay with total flavonoid content of *Garcinia lateriflora blume* leaves extract. Asian Journal Pharmaceutical Clinic Resources 10(5):163-165. *https://doi.org/10.22159/ajpcr.2017.v10s5.23122*

Balkis A, Tran K, Lee YZ, Ng K (2015). Screening flavonoids for inhibition of acetylcholinesterase identified baicalein as the most potent inhibitor. Journal of Agricultural Science 7:26-35. *https://doi.org/10.5539/jas.v7n9p26*

Bello KO, Nwozo SO, Oladele JO (2015). Hepatoprotective and antioxidant activities of the separate and combined administration of methanolic extract of *Adasonia digitata* and *Cochorous olitorious* leaves on rats. Ilorin Journal of Science 2(1):241-261.

Bhat AA, Shakeel A, Rafiq S, Farooq I, Malik AQ, Alghuthami ME, Alharthi S, Qanash H, Alharthy SA (2023). *Juglans regia* Linn.: A natural repository of vital phytochemical and pharmacological compounds. Life 13(2):380. *https://doi.org/10.3390/life13020380*

Borek C (2001). Antioxidant health effect of aged garlic extract. Journal of Nutrition 131:1010S1015S. *https://doi.org/10.1093/jn/131.3.1010S*

Chakraborty K, Paulraj R (2010). Sesquiterpenoids with free radical scavenging properties from marine macroalga *Ulva fasciata* Delile. Food Chemistry 122:31-41. *https://doi.org/10.1016/j.foodchem.2010.02.012*

Cheeseman KH, Holley AE (1993). Measuring free radicals reaction in vivo. British Medical Bulletin 49:491-505. *https://doi.org/10.1093/oxfordjournals.bmb.a072626*

Cohen D, Dembiec D, Marcus J (1970). Measurement of catalase activity in tissue extracts. Annals Biochemistry 34:30-38. *https://doi.org/10.1016/0003-2697(70)90083-7*

Deodhar M, Black DS, Kumar M (2007). Acid catalyzed stereoselective rearrangement and dimerization of flavenes: synthesis of dependensin. Tetrahedron 63:5227-5235. *https://doi.org/10.1016/j.tet.2007.03.173*

Ebrahimzadeh MA, Nabavi SF, Nabavi SM (2009). Antioxidant activities of methanol extract of *Sambucus ebulus* L. flower. Pakistan Journal of Biological Sciences PJBS 12(5):447-450. *https://doi.org/10.3923/pjbs.2009.447.450*

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemistry Pharmacology 7:88-95. *https://doi.org/10.1016/0006-2952(61)90145-9*

Gill SS, Mamdani M, Naglie G (2005). A prescribing cascade involving cholinesterase inhibitors and anticholinergic drugs. Archives of Internal Medicine 165(7):808-813. *https://doi.org/10.1001/archinte.165.7.808*

Guo AJY, Xie HQ, Choin RCY (2010). Galangin, a flavonol derived from *Rhizoma alpiniae officinarum,* inhibits acetylcholinesterase activity *in vitro.* Chemico-Biological Interactions 187(3):246-248. *https://doi.org/10.1016/j.cbi.2010.05.002*

Gupta A, Behl T, Panichayupakaranan P (2019). A review of phytochemistry and pharmacology profile of *Juglans regia*. Obesity Medicine 16:100142. *https://doi.org/10.1016/j.obmed.2019.100142*

Halliwell BG (1993). Free Radicals in Biology and Medicine. Oxford: Clarendon, pp 419.

Hayes D, Angove MJ, Tucci J, Dennis C (2016). Walnuts (*Juglans regia*) chemical composition and research in human health. Critical Reviews in Food Science and Nutrition 56:1231-1241. *https://doi.org/10.1080/10408398.2012.760516*

Heinrich M, Lee Teoh H (2004). Galanthamine from snowdrop-the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. Journal of Ethnopharmacology 92(2):147-162. *https://doi.org/10.1016/j.jep.2004.02.012*

Helou R, Rhalimi M (2010). Cholinesterase inhibitors and the risk of pulmonary disorders in hospitalized dementia patients. Journal of Population Therapeutics and Clinical Pharmacology 17(3);e379-389. *https://pubmed.ncbi.nlm.nih.gov/21041864/*

Hogan DB (2014). Long-term efficacy and toxicity of cholinesterase inhibitors in the treatment of Alzheimer disease. The Canadian Journal of Psychiatry 59(12):618-623. *https://doi.org/10.1177%2F070674371405901202*

Howes LG (2014). Cardiovascular effects of drugs used to treat Alzheimer's disease. Drug Safety 37(6):391-395. *https://doi.org/10.1007/s40264-014-0161-z*

Howes MJ, Houghton PJ (2003). Plant used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacology Biochemistry and Behavior 75:513-527. *https://doi.org/10.1016/s0091-3057(03)00128-x*

Katalinic M, Rusak G, Barovic JD (2010). Structural aspects of flavonoids as inhibitors of human butyrylcholinesterase. European Journal of Medicinal Chemistry 45(1):186-192. *https://doi.org/10.1016/j.ejmech.2009.09.041*

Khan MTH, Orhan I, Şenol FS (2009). Cholinesterase inhibitory activities of some flavonoid derivatives and chosen xanthone and their molecular docking studies. Chemico-Biological Interactions 181(3):383-389. *https://doi.org/10.1016/j.cbi.2009.06.024*

Kim JW, Minamikawa T (1997). Hydroxyl radical-scavenging effects of spices and scavengers from brown mustard (*Brassica nigra*). Bioscience, Biotechnology Biochemistry 61(1):118-123. *https://doi.org/10.1271/bbb.61.118*

Kim JY, Lee WS, Kim YS (2011). Isolation of cholinesterase-inhibiting flavonoids from *Morus lhou.* Journal of Agricultural and Food Chemistry 59(9):4589-4596. *https://doi.org/10.1021/jf200423g*

Knapp MJ, Knopman DS, Solomon PR, Pendlebury WW, Davis CS, Gracon SI (1994). A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. JAMA: The Journal of the American Medical Association 271(13):985-991. *https://doi.org/10.1001/jama.1994.03510370037029*

Kumar A, Sidhu J, Goyal A, Tsao JW (2022). Alzheimer Disease. Retrieved 2022 June 5 from: *https://www.ncbi.nlm.nih.gov/books/NBK499922/*

Liu RH (2013). Dietary bioactive compounds and their health implications. Journal of Food Science 78:A18-A25. *https://doi.org/10.1111/1750-3841.12101*

Marcocci L, Maguire JJ, Droy-Lafaix MT, Packer L (1994). The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. Biochemical and Biophysical Research Communications 201:748e755. *https://doi.org/10.1006/bbrc.1994.1764*

Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Fazelian M, Eslami B (2009). *In vitro* antioxidant and free radical scavenging activity of *Diospyros lotus* and *Pyrus boissieriana* growing in Iran. Pharmacy Magazine 5(18):122.

Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Hamidinia A, Bekhradnia AR (2008). Determination of antioxidant activity, phenol and flavonoids content of *Parrotia persica* Mey. Pharmacology Online 2(9):560-567.

Oladele JO, Oyeleke OM, Oladele OT, Oladiji AT (2021b). Covid-19 treatment: Investigation on the phytochemical constituents of *Vernonia amygdalina* as potential Coronavirus-2 inhibitors. Computational Toxicology 18:100161. *https://doi.org/10.1016/j.comtox.2021.100161*

Oladele JO, Ajayi EIO, Oyeleke OM, Oladele OT, Olowookere BD, Adeniyi BM, Oyewole OI, Oladiji AT (2020b). A systematic review on COVID-19 pandemic with special emphasis on curative potentials of medicinal plants. Heliyon 6:1-17. *https://doi.org/10.1016/j.heliyon.2020.e04897*

Oladele JO, Anyim JC, Oyeleke OM, Olowookere BD, Bamigboye MO, Oladele OO, Oladiji AT (2021). *Telfairia occidentalis* mitigates dextran sodium sulphate-induced ulcerative colitis in rats via suppression of oxidative stress, lipid peroxidation and inflammation. Journal of Food Biochemistry 45(9):1-12. *https://doi.org/10.1111/jfbc.13873*

Oladele JO, Oladele OT, Oyeleke OM (2021). Possible health benefits of polyphenols in neurological complications associated with COVID-19. Acta Facultatis Medicae Naissensis 38:294-309. *https://doi.org/10.5937/afmnai38-30190*

Oladele JO, Oyeleke OM, Oladele OT, Olaniyan MD (2020). Neuroprotective mechanism of *Vernonia amygdalina* in a rat model of neurodegenerative diseases. Toxicology Report 7:1223-1232. *https://doi.org/10.1016/j.toxrep.2020.09.005*

Oladiji AT, Oladele JO (2023). Spices as potential human disease panacea. Chapter 16. In: Kambizi L, Bvenura C (Eds). Sustainable Uses and Prospects of Medicinal Plants. CRC. *https://doi.org/10.1201/9781003206620-16*

Osher E, Gary W, Rona L, Karen T (2006). Naftali stern. The 5-lipoxygenase system in the vasculature: Emerging role in health and disease. Molecular and Cellular Endocrinology 252:201-206. *https://doi.org/10.1016/j.mce.2006.03.038*

Owokotomo IA, Ekundayo O, Abayomi TG, Chukwuka AV (2015). In-vitro anti-cholinesterase activity of essential oil from four tropical medicinal plants. Toxicology Reports 2:850-857. *http://dx.doi.org/10.1016/j.toxrep.2015.05.003*

Oyedapo OO, Akinpelu BA, Akinwunmi KF, Adeyinka MO, Sipeolu FO (2010). Red blood cell membrane stabilizing potentials of extracts of Lantana camara and its fractions. International Journal of Plant Physiology and Biochemistry 2(4):46-51.

Oyeleke OM, Bamigboye MO, Olowookere BD, Alabi KE, Oladele SK, Oladele JO (2021). Comparative studies on phytochemical constituents and *in vitro* antioxidant activities of methanol and ethylacetate extracts of *Talinum triangulare*. Journal of Basic and Applied Research in Biomedicine 7(1):18-23.

Oyewole OI, Oladele JO, Oladele OT (2017). Methanolic leaf extract of *Ficus exasperata* leaf attenuates arsenate-mediated hepatic and renal oxidative stress in rats. Research Journal of Health Sciences 5(2):115-123. *https://doi.org/10.4314/rejhs.v5i2.7*

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice Evans C (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26:1231-1237. *https://doi.org/10.1016/s0891-5849(98)00315-3*

Reuter S, Subash CG, Madan MC, Bharat B (2013). Aggarwal, oxidative stress inflammation, cancer: how are they linked? Free Radical Biology and Medicine 49:1603-1616. *https://doi.org/10.1016/j.freeradbiomed.2010.09.006*

Shahi Z, Sayyed-Alangi SZ, Najafian L (2020). Effects of enzyme type and process time on hydrolysis degree, electrophoresis bands and antioxidant properties of hydrolyzed proteins derived from defatted *Bunium persicum* Bioss. press cake. Heliyon 6(2):e03365. *https://doi.org/10.1016/j.heliyon.2020.e03365*

Tamura BK, Masaki KH, Blanchette P (2007). Weight loss in patients with Alzheimer's disease. Journal of Nutrition for the Elderly 26(3):21-38. *https://doi.org/10.1300/J052v26n03_02*

Voet D, Voet JG (1995). Serine proteases. In: Biochemistry. John Wiley & Sons, Hoboken, NJ, USA, 2nd edition.

Wszelaki N, Kuciun A, Kiss A (2010). Screening of traditional European herbal medicines for acetylcholinesterase and butyrylcholinesterase inhibitory activity. Acta Pharmaceutica 60:119-128. *https://doi.org/10.2478/v10007-010-0006-y*