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Article

Phytochemical profile, antioxidant and anti-inflammatory activities of *Ephedra alata* Decne growing in south Algeria

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Abstract: *Ephedra alata* Decne. (Ephedraceae) is a medicinal species commonly used to treat cancers, respiratory diseases, fever, and hypertension. The present study aimed to establish a phytochemical profile, evaluate the antioxidant potential and estimate the anti-inflammatory activities of E alata. Aqueous and methanolic extracts of *E. alata* aerial parts were phytochemically investigated using standard methods. DPPH, phosphomolybdenum total antioxidant capacity and reducing power assays were used to evaluate the antioxidant activity. Antioxidant activity was determined using total antioxidant capacity, the scavenging activity of the stable DPPH free radical and ferric reducing antioxidant power assays. The anti-inflammatory activity was determined using egg albumin membrane denaturation and human red blood cells membrane stabilizing assays. The anti-inflammatory potential of *E. alata* extracts was evaluated using human red blood cells membrane stabilization, egg albumin and BSA albumin denaturation assays. Quinones, anthraquinones, steroids, phytosteroids, phenols, terpenoids, flavonoids, saponins, glycosides, Cardiac glycosides, reducing sugars and anthocyanins were present in the E. alata's aqueous extract, in addition to coumarins and proteins in the methanolic extract. The highest total phenolic and flavonoid content was recorded in the aqueous extract with 8.66 ±0.09 mg GA/g and 248.04 ±1.47 mg Q/g, respectively. On the other hand, *E. alata* methanolic extract had the highest tannin content of 62.12 ±0.10 mg C/g. The best radical scavenging activity (IC_{50} = 4.63±0.00 mg/ml) and total antioxidant capacity were exhibited by the E. alata aqueous (7.35±0.12 mg/ml AAE), whereas the methanolic extract possessed the highest reducing power activity (1.81±0.00 mg AAE/ml). Regarding the anti-inflammatory activities, *E. alata* methanolic extract exerted the highest HRBC stabilization of 34.72 ±0.08% whereas the aqueous extract exhibited the highest bovine serum and Egg albumin denaturation inhibition of 99.22 ±0.02% and 73.31 ±0.90, respectively. Taken together, our results suggest that *E. alata* aerial parts aqueous and methanolic extracts can be utilized as future antioxidants and anti-inflammatory ethnomedicines owing to their rich bioactive molecules content.

Keywords: Ephedra alata; antioxidant activity; anti-inflammatory activity; phytochemical screening

1. Introduction

Algeria is one of the richest Arabic and African countries regarding medicinal plants with 3164 species[1], they are widely used by Algerians who still relay on traditional healers for therapy[2]. The World Health Organization (WHO) reported that 80% of the emerging world population relies on traditional herbal medicine for their healthcare and

an uprise in their utilization in the developed countries[3] with a worldwide annual market value of US$60 billion[4]. Studies have reported that 60% of new drugs introduced between 1981 and 2002 are derived from plants, even today, the majority of anticancer drugs are mainly derived from plants[3,5].

Ephedra is one of the largest genera of the *Ephedraceae* family, it consists of 69 species mainly distributed in semi-arid environments, although some species are distributed through few Neotropical countries[6,7]. Ephedras are traditionally used to treat allergies, bronchial asthma, chills, colds, coughs, edema, fever, flu, headaches and nasal congestion[8], they have been used in the US since the 20th for weight loss and to treat syphilis and gonorrhea and in China for more than 5000 years and Ayurvedic medicine as a stimulant and an anti-asthmatic while Pharmacological trials on crude extracts, fractions and few isolated compounds of Ephedra species in vitro and in vivo demonstrated anti-inflammatory, anticancer, antibacterial, antioxidant, hepatoprotective, anti-obesity, antiviral, and diuretic activity[9]. However due to its side effects caused by misuse and the high alkaloid content (ephedrine and pseudoephedrine) the ephedra use has been controlled[8], interestingly, among all the *Ephedra* species, only *Ephedra alata* is known for its low alkaloid content.

Ephedra alata, commonly known in Algeria as Alenda[10], is a medicinal plant of the family Ephedraceae, it is a perennial and xerophytic (adapt to any environment to survive) gymnosperm and dioecious (has distinct male and female organisms which excludes self-fertilization) shrub with erect non climbing stems and short leaves that are united towards base and yellowish-green bracts that flowers in july[11,12] and have a strong pine like smell[13,14], the shrub grows in the temperate, subtropical and dry environments and distributed in the north African countries: Algeria, Tunisia, Morocco, Egypt, Chad, Mauritania and Mali; in Asia: China, Palestine, Lebanon, Jordan, Saudi Arabia, Iran and Iraq and in Europe: Spain[15,16]. *E. alata* is used in traditional Russian medicine for treating respiratory disorders and rheumatism, the plant is also used in traditional Chinese Pharmacopoeia against cough, hay fever, cold, chills, asthma, allergies and edema[11,17] , while Algerians and Tunisians use it as anti-cancer treatment[18] and it has been reported to decrease the side effects of chemotherapy[19,20]. The primary active molecules in *E.alata* are the ephedrine alkaloids which have major side effects if used in high doses such as adverse cardiovascular and cerebrovascular events which explains why the FDA has banned any drugs that contains the ephedrine alkaloids[15,18,21,22], The major compounds detected in *E. alata* growing in Algeria were isoflavones and flavonol derivatives with hydroxypuerarin isomer as a lead molecule[2]

The present study aimed to establish a phytochemical profile, evaluate the antioxidant potential and estimate the anti-inflammatory activities of *E. alata* growing in Algeria.

2. Results

2.1. Extraction yield

According to our results, the highest extraction yield (38%) was obtained with the *E. alata* aqueous extract while the methanolic extract had the lowest extraction yield of

15% (Table 1). This difference could be due to differences in solvent polarities which extract different types and amounts of metabolites.

Table 1. Extraction yield of *E. alata* extracts

	Aqueous extract	**Methanolic extract**
Plant powder weight (g)	20	20
Solvent (ml)	200 (x3)	200
Crud extract weight (g)	7.6	3
Extraction yield %	38	15

2.2. Phytochemical screening

As shown in table 2, the phytochemical analysis conducted on the *E. alata* aqueous extract of revealed the presence of quinones, anthraquinones, steroids, phytosteroids, phenols, terpenoids, flavonoids, saponins, glycosides, cardiac glycosides, reducing sugars and anthocyanins. The methanolic extract showed the presence of quinones, coumarins, steroids, phytosteroids, phenols, terpenoids, flavonoids, saponins, glycosides, cardiac glycosides, reducing sugars, proteins and anthocyanins. Surprisingly, both extracts did not contain alkaloids unlike other Ephedra species.

Table 2. Phytochemical screening of E. alata aqueous and methanolic extracts

Phytochemical	**Aqueous extract**	**Methanolic extrat**
Quinones	++	++
coumarins	-	++
Anthraquinones	+	-
Steroid	+	++
Phyto-steroids	+	++
Phenols	++	++
Terpenoids	++	++
Phlobotannins	-	-
Glucosides	-	-
Flavonoids	+	+
Alkaloids	-	-
Saponins	+	+
Cardiac glycoside	+	+

Resin	-	-
Reducing sugars	++	+
Proteins / amino acid	-	+
anthocyanin	++	++
Iridoids	-	-

+ Present, ++ Highly present, - Absent

2.3. Total phenolic, flavonoid and tannin contents

Our results (table 3) revealed that *E. alata* aqueous extract had the highest total phenolic and flavonoid content of 8.66 ±0.09 mg GA/g and 248.04 ±1.47 mg Q/g, respectively. The highest tannin content of 62.12 ±0.108 mg C/g was recorded for the methanolic extract whereas that of the aqueous extract was 34.68 ±0.06 mg C/g. On the other hand, the methanolic extract had the lowest phenolic and flavonoid contents of 3.16 ±0.004 mg GA/g and 154.54 ±0.53 mg Q/g, respectively.

Table 3. Total phenolic, flavonoid and tannin content of *E. alata* extracts

	Total phenolic content (mg GA/g) ±SD	**Condensed tannins content (mg C/g) ±SD**	**Total flavonoids content (mg Q/g) ±SD**
Aqueous extract	8.66 ±0.092	34.68 ±0.068	248.04 ±1.471
Methanolic extract	3.16 ±0.004	62.12 ±0.108	154.54 ±0.535

2.4. Antioxidant activities

2.4.1. DPPH radical scavenging activity

According to our DPPH radical scavenging activity results (figure 2), there was a significant difference between the *E. alata* aqueous and methanolic extract ($p< 0.05$) and between the methanolic extract and the ascorbic acid control ($p< 0.01$). Table 4 and As shown in figure 1, the aqueous extract had the best free radical scavenging activity with an IC_{50} of 4.638±0.002 mg/ml, while the methanolic extract presented a low scavenging activity with a high IC_{50} of 20.943±2.903 mg/ml. The IC_{50} of the ascorbic acid used as a standard was found to be 2.786±0.019 mg/ml.

Table 4. IC_{50} of *E. alata* extracts (Free radical scavenging activity)

Extract	**Aqueous extract**	**Methanolic extract**	**Ascorbic acid**
IC_{50} (mg/ml) ±SD	4.638±0.002	20.943±2.903	2.786±0.019

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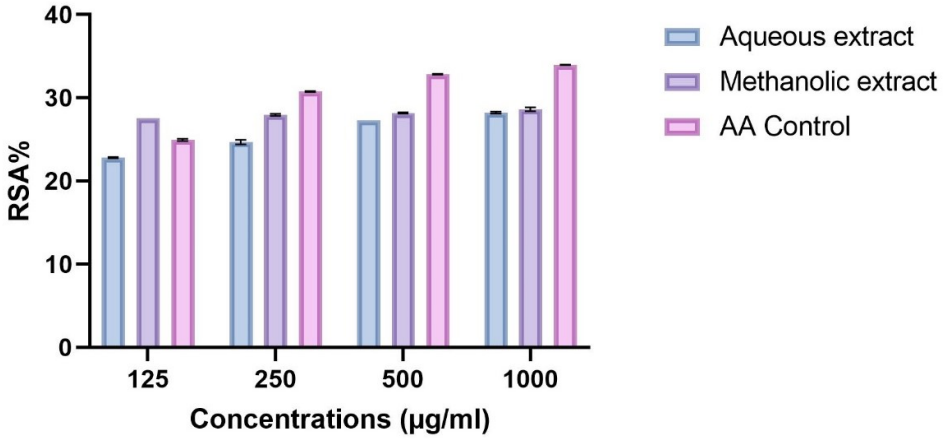

Figure 1. Radical scavenging activity of *E. alata* extracts

2.4.2. Total antioxidant activity

Our results (table 5) showed that *E. alata* aqueous extract exhibited the highest total antioxidant capacity (7.35±0.125 mg/ml AAE) followed by the methanolic extract with just a slight difference (7.3±0.125 mg/ml AAE). Statistically, there was no significant difference between both extracts. Interestingly, the total antioxidant capacity of both *E. alata* extracts was almost six-fold higher than that of ascorbic acid (figure 2).

Table 5. Total antioxidant capacity of *E. alata* extracts

Extract	Aqueous extract	Methanolic extract	Ascorbic acid control
TAC (mg/ml AAE) ±SD	7.35±0.125	7.3±0.025	1.27±0.23

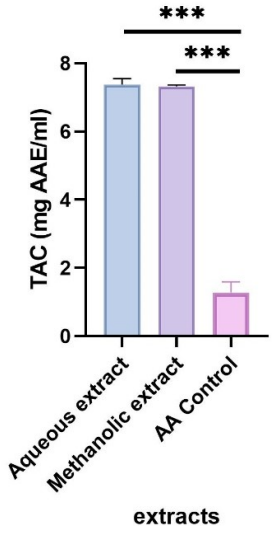

Figure 2. Total antioxidant capacity of *E. alata* extracts

*** Significant at $p < 0.001$

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2.4.3. Reducing power

The reducing power activity results showed that there was a dose-dependent increase in reducing power for both extracts (Figure 3). The *E. alata* methanolic extract possessed the highest reducibility (1.81±0.00 mg of AAE /ml of extract) at 1000µg/ml, while the aqueous extract had 1.36±0.02 mg AAE/ml at the same concentration. However, this difference was not statistically significant.

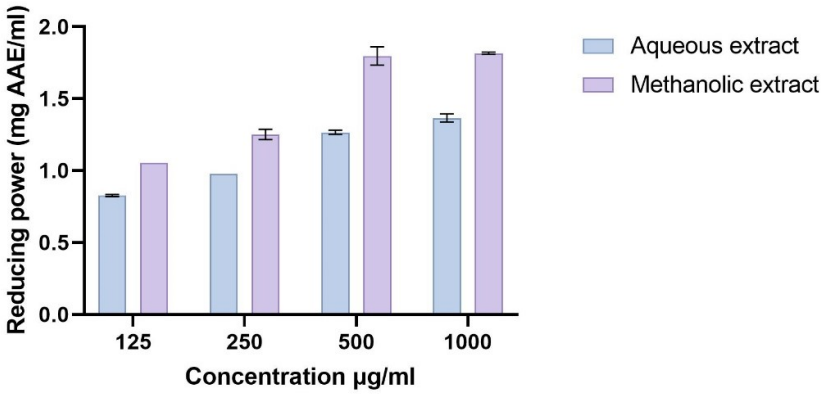

Figure 3. Reducing power of *E. alata* extracts

2.5. Antiinflammatory activities

2.5.1. HRBC membrane stabilization

As shown in figure 4, both *E. alata* extracts exhibited significant higher human red blood cells membrane stabilization when compared to that of the standard diclofenac sodium. This HRBC stabilization potential was not in a dose-dependent manner. Indeed, the *E. alata* methanolic extract had the highest HRBC stabilization of 34.72 ±0.08% at 125µg/ml whereas this potential decreased to 5.82±0.00% at 1000µg/ml. Regarding E. *alata* aqueous extract, the highest HRBC membrane stabilization of 18.31% was obtained at 500 µg/ml. When compared to the standard diclofenac sodium, both *E. alata* extracts at 125, 250, and 500 µg/ml exhibited higher stabilization activity. Nonetheless, at 1000µg/ml, stabilization activity of diclofenac sodium was the highest.

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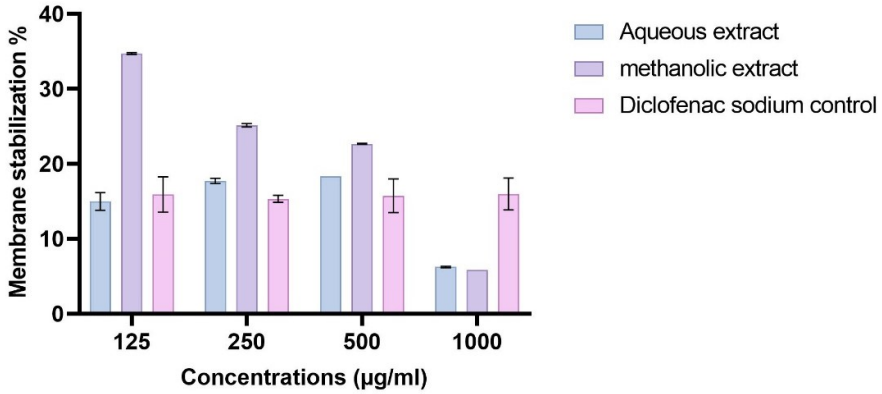

Figure 4. HRBC membrane stabilization activity of *E. alata* extracts

2.5.2. Protein denaturation inhibition activities

- Bovine Serum albumin denaturation inhibition

Bovine Serum albumin denaturation inhibition assay results (figure 5) indicate that the aqueous extract of *E. alata* had the highest inhibition percentage at 50 µg/ml (99.22 ±0.022%) even higher than the standard diclofenac sodium (97.65% ±0.440), while the methanolic extract had an inhibition activity of 89.74% ±0.60 at the same concentration.

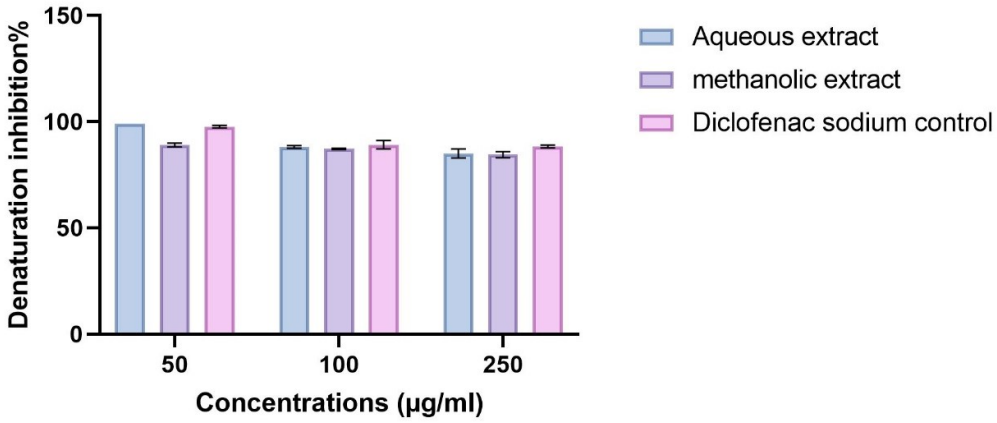

Figure 5. Bovine serum albumin protein denaturation inhibition activity of *E. alata* extracts

- Egg albumin denaturation inhibition

As table 10 highlights, the *E. alata* aqueous extract exerted the highest inhibition activity of protein denaturation at concentrations of 125 and 500 µg/ml (73.31 ± 0.906 and 72.02 ± 0.064%, respectively) followed by the methanolic extract at concentration of 1000 µg/ml (72.54% ±0.518). Although both extracts revealed high inhibition activities, none of them surpassed the standard diclofenac drug that had a high protein denaturation

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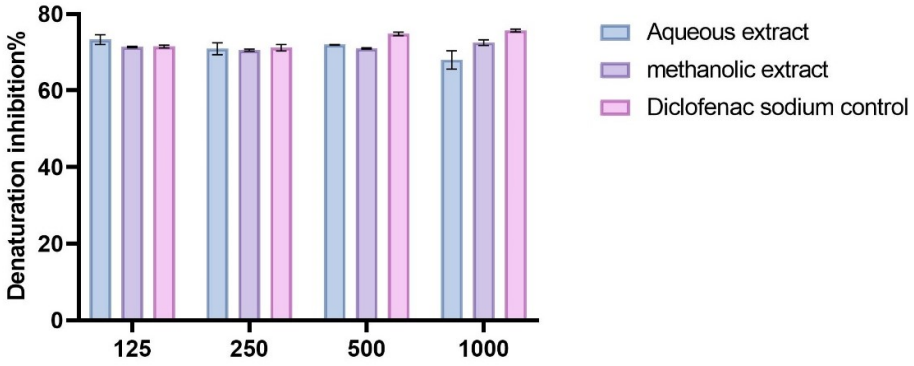
inhibition activity of 75.56% ± 0.259 and 74.87% ± 0.323 at concentration 1000 and 500 µg/ml, respectively.



2.1.1. Subsubsection

3. Discussion

This study was mainly carried out to establish the qualitative and quantitative phytochemical profile, and evaluate the antioxidant capacity and anti-inflammatory activities of *E. alata* growing in south Algeria. *E. alata* is a medicinal plant widely used in Asia and North Africa for its antioxidant, anti-inflammatory, anti-arthritic and anticancer potentials owing to its rich content of secondary metabolites.[48-50]

Our results revealed the presence of various bioactive phytochemicals in both *E. alata* extracts. Our findings are consistent with those of Jaradat et al (2015)51 except for alkaloids. Interestingly, we showed that both *E. alata* extracts did not contain alkaloids, which confirms the results reported by Ibragic and Sofić (2015) [21] indicating the lack of alkaloids in *E. alata* unlike other Ephedra species.

Furthermore, the aqueous extract had the highest phenolic and flavonoid content while the methanolic extract had the highest tannin content. When compared to *E. alata* collected from Tatouine Sahara (Tunisia) which had a TFC of 2.8 ± 0.0 mg Q/g and a TPC of 53.3±0.1mg GA/g (Ibragic and Sofić, 2015)[21], *E. alata* collected from The Algerian Sahara (Adrar) had higher flavonoid content and lower phenolic content. This difference may be due to various factors such as the harvesting location and date, soil properties, rainfall, plant storage and extraction methods[18].

Phenolics are major antioxidants that were detected in both aqueous and methanolic *E. alata* extracts. We therefore evaluated the antioxidant activity of *E. alata* extracts using different assays (DPPH, TAC and reducing power). According to the DPPH radical scavenging activity and the total antioxidant capacity results, the *E. alata* aqueous extract had higher antioxidant potentials compared to the methanolic extract which could be explained by its higher phenolic and flavonoid content. Nevertheless, the results reported by Jaradat et al[51] revealed a better antioxidant activity with lower IC_{50} of Palestinian *E. alata*, which can be attributed to their alkaloids content, considered as important antioxidants. Our phytochemical results revealed that Algerian *E. alata* did not

contain alkaloids, which can be associated with a safe toxicological profile since Ephedra alkaloids (ephedrine and pseudoephedrine) are responsible for toxicity[18,21]. However, the Reducing power assay showed that the methanolic extract exhibited higher reducing power in all different concentrations. In correlation with our results, Al-Rimawi et al. (2017)[52] reported that the reducing power of Palestinian *E. alata* significantly changed according to the solvent polarity.

Since human erythrocytes' membrane has similar structure to the lysosomal membrane, HRBC membrane stabilization assay is considered as a measure of anti-inflammatory activity of the plants extracts. According to our results, the E. alata methanolic extract had better anti-inflammatory activity than the standard widely used Diclofenac sodium, whereas the aqueous extract exhibited a membrane stabilizing activity similar to this standard NSAID. To further confirm our results, protein denaturation inhibition activity assay was conducted considering that protein denaturation is an indirect cause of inflammation through cell destruction or mutilation. Therefore, substances able to inhibit protein denaturation can assumably inhibit inflammation. Both bovine serum albumin denaturation and egg albumin denaturation assays indicated that the aqueous extract had the highest protein denaturation inhibition percentage that was comparable to the anti-inflammatory potentials of diclofenac sodium. Taken together, our results reveal that both studied extracts have significant anti-inflammatory abilities specially the methanolic extract which correlate with the results stated by Kmail et al (2017)[53] and the plants traditional uses in treating inflammation and inflammation-related diseases such as cancer. Indeed, *E. alata* was demonstrated to be able to inhibit the release of pro-inflammatorymediators such as the pro-inflammatory cytokines IL-1β, IL- TNF-α,IL- 6, IL 8, and IFN-γ by down-regulating Anti-inflammatory cytokines that includes IL-1receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13[53].

In conclusion, the present study showed that both aqueous and methanolic extracts of E. alata aerial parts contain various phytochemicals. Furthermore, *E. alata* aqueous extract possessed the highest total phenolic and flavonoid content whereas the highest tannin content was found in the methanolic extract. Interestingly, all *E. alata* extracts exhibited promising antioxidant and anti-inflammatory properties. Our results indicate that both extracts can be utilized as future antioxidants and anti-inflammatory ethnomedicines. Further *in vivo* studies are needed to validate their pharmacological properties.

4. Materials and Methods

Plant material and extracts preparation

Ephedra alata whole plant was collected from Adrar (South Algeria) in January 2020. Botanical identification was performed at the department of biology (University of Mascara, Algeria). Aerial parts were collected, cleaned, dried in semi shaded area for few weeks, and then finely grounded and stored in containers in dark.

Preparation of *E. alata* aqueous extract

The aqueous extract of *E. alata* aerial parts was prepared as follows: 20g of dried plant powder were decocted in 200 ml of water at 100°C for 10 mins, cooled to room temperature, and then filtered. This procedure was repeated twice [25-27]. After concentration, the crude aqueous extract was stored at +4°C until use.

Preparation of *E. alata* methanolic extract

The methanolic extract of *E. alata* aerial parts was prepared as follows: 20g of dried plant powder were macerated in 200 ml of methanol for one week at room temperature. After filtration, methanol was evaporated[25-27] and the obtained methanolic extract was stored at +4°C until use.

The extraction yield (%) was calculated using the following formula [28]:

$$Extraction\ yield\ (w/w\%) = \frac{Weight\ of\ dried\ crude\ extract}{Weight\ of\ loquat\ powder}$$

Phytochemical screening – The phytochemical profile of E. alata was performed using standard procedures described [29, 30] with slight modifications.

Phytochemical quantitative analysis

Total phenolic content–Total phenolic content was determined using the Folin-Ciocalteu assay[31]. In brief, 100 µl of each extract and 500µl of 1% Folin-Ciocalteu reagent were added to 400 µl of 7.5% Sodium carbonate solution (w/v), mixed using a vortex, and then incubated for 10 min in the dark at room temperature. The absorbance was then measured at 760nm[32]. The phenolic content was calculated as gallic acid equivalents GAE/g of dry plant material. Values were determined in triplicate.

Total flavonoids content– The determination of the total flavonoid content of the extracts was carried out using the aluminum chloride colorimetric method[33]. 1 ml of the extract was added to 0.3ml sodium nitrite $NaNO_3$and incubated for 5 min, then 0.3ml of Aluminum trichloride $AlCl_3$was added and 2ml of sodium hydroxide NaOH after 6 min. The absorbance was measured at 510 nm using a UV-Visible spectrophotometer against a blank. A calibration curve was performed in parallel using different concentrations of quercetin under the same conditions as a positive control[34]. The results were expressed as mg Quercetin/g of dry extract. Values were determined in triplicate.

Condensed Tannins Content– The condensed tannins content of the extracts was analyzed using the vanillin-HC1 method of Broadhurst and Jones [35]. 0.5ml of the extract was homogenized with 3ml of 3% vanillin/methanol (w/v) and 1.5ml HCl, and then

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incubated at room temperature for 15 min. The absorbance was measured at 500nm. A calibration curve of different concentrations of catechin was performed under the same operating conditions. The results were expressed as mg catechin/g of dry extract. Values were determined in triplicate.

Antioxidant activity

DPPH radical scavenging activity– The DPPH radical scavenging activity was evaluated as described by Brand-Williams et al[36]. Four concentrations of each extract were prepared. 2 ml of methanolic DPPH solution (4mg/100ml) freshly prepared was added to 1 ml of the extract and then incubated in the dark at room temperature for 16 min. The absorbance was measured at 517 nm using a UV-Visible spectrophotometer[37], against the methanolic solution of DPPH as a control, and the extraction solvent as a blank. Ascorbic acid was used as positive control[38].The DPPH free radical scavenging activity is indicated through the degree of colour transformation from purple to yellow indicates [39,40]which is expressed as percentage scavenging using the formula:

$$DPPH\ inibitiona\ ctivity\ (\%) = \frac{A1-A2}{A1} \times 100$$

A1: absorbance of the control (DPPH solution) without extract

A2: absorbance after the addition of the extract

The radical scavenging activity (%) data was then plotted against concentration in a graph and IC_{50} (half-maximal inhibitory concentration) value was calculated by linear regression analysis.

Total antioxidant capacity (TAC) assay (phosphomolybdate assay)– Test tubes containing 200µl of extract (1mg/ml) and 2 ml of phosphomolybdate reagent were placed in a water bath at 95°C for 90 minutes then cooled down and the absorbance level was measured at 695 nm against a blank of methanol or distilled water instead of plant sample[41]. A calibration curve was performed using different concentrations (62,125, 250,500, 1000µg/mL) of ascorbic acid to calculate Ascorbic acid equivalent[39,40]. Values were expressed as mg AAE/ml of extract.

Reducing power (RP) assay– The reducing power was measured as described by Oyaizu[42]. In brief, tubes containing different concentrations of the extract, 2.5 mL phosphate buffer (0.2 M, pH 6.6), and 2.5 mL potassium hexacyanoferrate (1%) were incubated in a water bath for 20 min at 50°C. Then 2.5 mL of Trichloroacetate (10%) was added, and the mixture was centrifuged at 3000rpm for 10 min. Then, 2.5 mL distilled water and 0.5 mL $FeCl_3$ solution (0.1%) were mixed with 2.5ml of the supernatant and the absorbance was measured at 700 nm against a blank solution prepared by substituting the same amount of diluted extract with its vehicule[40]. Different concentrations (125, 250, 500, 1000 µg/mL) of ascorbic acid were used as standard and the results were expressed in mg AAE/ml extract.

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Anti-inflammatory activity

HRBC membrane stabilizing assay – Human red blood cells membrane stabilization assay was carried out as described by Sunmathi et al. (2016)[43]**.** Type O^+ Blood was collected in Heparin tubes from healthy volunteers who have not been taking any NSAIDS for the past two weeks, and centrifuged at 3000rpm for 10 min. The pellet was washed with equal volume of PBS (pH = 6.3) for three times. The human red blood cells were re-suspended in PBS (10% v/v). 1ml of various concentrations of the extracts (125, 250, 500, 1000µg/ml) was added to 1 ml of phosphate buffer, 2 ml hypo saline (0.25% w/v NaCl) and 0.5 ml of HRBC suspension. The reaction mixture was incubated at 37°C for 30 min and then centrifuged at 300rpm for 20 min. The haemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm against a blank of the extract's vehicle using the following formula:

$$\text{Membrane stabilization (\%)} = 100 - \left(\frac{A1-A2}{A1} \times 100\right)$$

A_1: Absorbance of hypotonic-buffered saline solution alone

A_2: Absorbance of test sample

Different concentrations of sodium diclofenac were used as a reference drug for this assay.

Albumin denaturation activity– The denaturation of albumin protein leads to the formation of antigens which initiate type III hypersensitive reaction leading to inflammation[44]. Thus, the plant extracts able to inhibit protein denaturation are considered capable of inhibiting inflammation. Since nowadays sodium diclofenac is commonly prescribed as an anti-inflammatory drug, it was used as a reference standard for both assays of this activity[45]:

Bovine serum albumin (BSA) denaturation assay – 0.45mL of BSA (0.5% w/v aqueous solution) and 50µl of different concentrations of the extract (125,250,500,1000µg/ml) were mixed, incubated at 37°C for 20 min, and then heated at 57°C for 3 min. 2.5mL of PBS (pH = 6.6) was added and absorbance was measured at 255 nm against a blank of the extract's solvent. Diclofenac sodium of various concentrations was used as a standard for this assay. 0.45mL of 0.5% BAS solution and 50µl distilled water constituted the control solution[43,46]. The percentage inhibition of protein denaturation was calculated using the following equation:

$$\textit{inhibition of protein denaturation (\%)} = 100 - \left(\frac{A1-A2}{A1} \times 100\right)$$

A_1: Absorbance of the test solution

A_2: Absorbance of control

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Egg albumin denaturation assay – The membrane denaturation assay was carried out as described by Shelke et al. (2020)[47]. 2ml of the extract (125, 250, 500, 1000 µg/ml) was mixed with 0.2mL of egg albumin (0.5% w/v aqueous solution) and 2.8 PBS (pH = 6.4), and incubated at 37°C for 15 min. The mixture was then heated at 70°C for five minutes in a water bath. Aabsorbance was measured at 660 nm against the solvent as a blank. for the control, distilled water was used instead of the extracts. A calibration curve was performed in parallel under the same operating conditions using sodium diclofenac as a positive control[43]:

$$inhibition \text{ of protein denaturation } (\%) = \frac{A2-A1}{A2} \times 100$$

A_1: Absorbance of the test sample

A_2: Absorbance of the Control

Statistical analysis – Data were presented as means ± SD. Statistical significance ($p < 0.05$, $p < 0.01$, $p < 0.001$and $p < 0.0001$) was assessed by one-way analysis of variance (ANOVA) for the antioxidant activity and two-way analysis of variance (ANOVA) coupled with Tukey's post-hoc test.

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