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Antioxidant, Anti-inflammatory and skin-aging Enzymes Inhibition potential of *Asystasia vogeliana* leaf n-hexane Extract and its Dechlorophyllized Equivalent

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Abstract

Purpose: The study investigated the antioxidant, anti-inflammatory, photoprotective and skin-aging retardation activities of *Asystasia vogeliana* leaf n-hexane extract (AVHE) and its dechlorophyllized equivalent (DAVHE). **Methods:** The total phenolics, flavonoids, vitamin A and vitamin E contents of the extracts were quantified. Antioxidant (DPPH scavenging, ferric reducing antioxidant power, total antioxidant capacity, lipid peroxidation and metal chelating inhibition), anti-inflammatory (lipoxygenase, trypsin and protein denaturation inhibition), photoprotective (SPF and UV blocking) and skin-aging enzymes (collagenase and tyrosinase inhibition) activities were evaluated using standard methods. The components of the most potent extract were identified via GC-MS technique. **Results:** AVHE and DAVHE possess slight concentrations of total phenolics (9.89±0.21 mg GAE/g and 1.43±0.08 mg GAE/g),and flavonoids (0.68±0.00 mg GAE/g, 2.13±0.00 mg GAE/g); AVHE exhibited significantly higher vitamin A and E content (87.29±0.84 mg RE/g, 12.04±0.11 mgTE/g) than DAVHE (41.29±0.74 mgRE/g, 7.31±0.82 mg TE/g). Both extracts showed moderate antioxidant activity. AVHE demonstrated higher trypsin inhibition (81.44%±0.83), protein denaturation inhibition (62.07%±0.00) and lipoxygenase inhibition (IC_{50}=0.458±0.041 mg/ml). Both extract had similar collagenase and tyrosinase inhibitory activities. AVHE showed a significantly higher sun photoprotective activity (46.35±0.55) than DAVHE (28.92±5.13) and a commercial sunscreen (11.65±0.01). GC-MS analysis identified major constituents in AVHE, including 9,12,15-octadecatrienoic acid, (Z,Z,Z); n-hexadecanoic acid; 1-octadecyne and phytol. **Conclusion:** *A. vogeliana* leaf n-hexane extract is a promising candidate for skin care formulation. However, dechlorophyllization slightly reduces its efficacy.

Keywords: *Asystasia vogeliana*, Antioxidant, Anti-inflammatory, Skin aging enzymes, Photoprotective

Introduction

Skin aging is a multifaceted biological process influenced by a range of genetic and environmental factors which causes gradual decline in the skin structural integrity and ability to perform its normal functions (Zhang & Duan, 2018; Korać & Khambholja, 2011). Chronic exposure to UV radiation from sunlight amongst other factors is one of the prominent extrinsic factors, which results in oxidative damage of key skin components including collagen, elastase and other matrix metalloproteins, lipids and DNA. Subsequently, this activates inflammatory pathways and some skin-aging related enzymes including collagenase and tyrosinase, thereby resulting in early skin aging (Pillai *et al*., 2005).

Recent research has increasingly focused on antioxidants,

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substances that can delay or mitigate oxidative stress-induced skin damage and aging (Feng *et al*., 2021). Plant-derived antioxidants are of particular interest due to their abundant availability and potent bioactive properties. Plants are known to produce a wide variety of photoprotective compounds which that can provide both antioxidant and anti-aging benefits (Lee, 2023; Ibrahim *et al*., 2022). This has driven a growing body of research towards the use of plant extracts in the development of natural skin care formulations. Chlorophyll, a key pigment in photosynthesis, is abundant in plant materials but presents challenges in extract applications due to its strong colouration and partial solubilty in polar solvents (Bohn *et al*., 2004). The presence of chlorophyll can limit the cosmetic appeal of plant extracts by imparting an undesirable green colour, thus necessitating the process of dechlorophyllization to enhance their usability in cosmeceutical formulations (Namal Senanayake, 2013). However, dechlorophyllization may also alter the chemical profile of plant extracts, potentially affecting their bioactive properties, including UV absorption and antioxidant properties (Olatunde *et al*., 2018).

Asystasia vogeliana (Benth), a lesser-studied member of the Acanthaceae family is a multipurpose medicinal plant used traditionally for treatments of various ailments such as hepatitis, malaria, gastric disorder and menstrual disorder (Ugwuanyi *et al*., 2020; Popoola *et al*., 2017). Studies have shown that certain species within the *Asystasia genus* (Acanthaceae family) possess skin-aging retardation properties (Barbaza *et al*., 2020), yet, the specific anti-aging potential of *A. vogeliana* remains underexplored. Given its traditional uses and phytochemical profile, this study aims to investigate antioxidant, anti-inflammatory, anti-collagenase, anti-tyrosinase as well as photoprotective activities of both dechlorophyllized and non-dechlorophyllized n-hexane extract of *A. vogeliana*, with the aim of assessing their suitability for inclusion in anti-aging formulations and to contribute new insights into the role chlorophyll and other bioactives in skin health.

Materials and Methods

1. Collection and identification of plant sample

Fresh samples of *A. vogeliana* leaf was obtained in August, 2020 at the the Natural History Museum garden, Obafemi Awolowo University (OAU), Ile-Ife, Nigeria. The samples were identified and authenticated at IFE Herbarium, Department of Botany, OAU, Ile-Ife, Nigeria.

2. Chemicals

The reagents utilized were products of several manufacturers including British Drug House (BDH) Chemicals Limited, Poole, England and Sigma-Aldrich, Inc. Sodium potassium tartrate, disodium hydrogen orthophosphate, copper (II) tetraoxosulphate (VI), thiobarbituric acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium hydroxide, bovine serum albumin, sodium dihydrogen phosphate, methanol, potassium ferrocyanide, trichloroacetic acid, ferric chloride, copper sulphate, acetic acid, sodium dodecyl sulphate, butanol, ascorbic acid, ammonium molybdate, sulphuric acid, Folin Ciocalteu's reagent, Sodium trioxocarbonate (IV), gallic acid, ethylene diamine tetracetic acid, sodium nitrite, quercetin, aluminium chloride, trypsin, Tris base, hydrochloric acid, casein, bovine serum albumin, 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA), piroxicam, collagenase, mushroom tyrosinase, L-3,4-dihydroxyphenylalanine (L-DOPA), Kojic acid, n-hexane, acetone, chloroform, tocopherol.

3. Preparation of extracts

Fresh leaves of *A. vogeliana* were cleaned, air-dried to a constant weight and ground into powder. The grounded leaf sample was divided into two portions. The first portion was dechlorophyllized using 80% acetone as described by Olatunde *et al*. (2018). 50 g of the first portion was mixed with 500 mL of 80% acetone and stirred continuously for 30 minutes for chlorophyll removal. Subsequently, the mixture was filtered. The residue was subjected to dechlorophyllization twice. The dechlorophyllized powder was air-dried at room temperature. The resulting powder was named "dechlorophyllized leaf powder" while the second portion of the leaf powder was named "non-dechlorophyllized leaf powder". The two portions of leaf were macerated separately in n-hexane in ratio 1:10 for 72 hours. The filtrates were collected and concentrated to dryness using a rotary evaporator (Buchi Rotavapour, vacuum pump V; Switzerland) under reduced pressure at 27℃ to obtain the *A. vogeliana* n-hexane extract (AVHE) and dechlorophyllized *A. vogeliana* n-hexane extract (DAVHE).

4. Estimation of chlorophyll content

The total chlorophyll content of AVHE and DAVHE were

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determined using a spectrophotometric method as reported by Roshanak *et al*. (2015) with slight moifications. The dry extracts were dissolved in n-hexane to obtain solutions with concentrations of 2.5 mg/mL, and the absorbance of these solutions were read at 663 nm and 645 nm using a spectrophotometer (S23A; Spectrumlab, England), with n-hexane serving as the blank. Subsequently, the chlorophyll a, b, and total chlorophyll content of the extracts were calculated using the equations provided below:

Chlorophyll a (mg/mL)=12.7 (A_{663})-2.69 (A_{645})

Chlorophyll b (mg/mL)=22.9 (A_{645})-4.68 (A_{663})

Total chlorophyll (mg/mL)=20.2 (A_{645})+8.02 (A_{663})

Where A_{663} is the absorbance at 663 nm and A_{645} is the absorbance at 645 nm.

5. Estimation of total phenolic content

Total phenolic content of AVHE and DAVHE were investigated through a modified method of the Folin-Ciocalteu's phenol reaction (Bode & Oyedapo, 2011; Singleton *et al*., 1999). The technique involved mixing the 0.2 mL of extract (1 mg/mL), with 0.8 mL of distilled water and 1.5 mL Folin-Ciocalteau's Phenol reagent. The assay mixtures were incubated for 15 minutes at room temperature. Afterwards, 10% (w/v) Na_2CO_3 (1.5 mL) was pipetted into the mixture and further incubated for 90 minutes in a dark condition at room temperature. Absorbance of the resultant reaction mixture was taken at 720 nm against reagent blank using a spectrophotometer (S23A; Spectrumlab, England, England). The total phenolics in the extracts were extrapolated from gallic acid calibration curve and presented in mg/g (GAE) gallic acid equivalent.

6. Estimation of total flavonoid content

The concentration of flavonoids in AVHE and DAVHE were determined via aluminum chloride reaction method (Sun *et al*., 1999). The procedure involved mixing 0.2 mL of extract with 2.8 mL of distilled water, 0.3 mL each of 10% (w/v) $AlCl_3$ and 5% (w/v) $NaNO_2$, and 4% NaOH (4 mL). The mixtures were allowed to stand at room temperature for 15 minutes, after which the absorbance were measured at 500 against reagents blank as a negative control using a spectrophotometer (S23A; Spectrumlab, England, England). The flavonoid content of the extracts was calculated and expressed in milligrams of quercetin equivalent per gram (mg/g QE).

7. Estimation of total vitamin A content

The vitamin A content of AVHE and DAVHE were determined according the standard method of the Association of Official Analytical Chemists (AOAC, 1990) as reported by Achikanu *et al*. (2013). Each extract (1 g) was macerated in 20 mL of n-hexane for 10 minutes. Afterwards, the upper hexane solution (3 mL) was pipetted into uncontaminated dry test tubes in triplicates. The mixture was later evaporated to dryness on a water bath. Acetic anhydride chloroform reagent (0.2 mL) and 50% w/v trichloroacetic acid (TCA) dissolved in chloroform (2 mL) were added to the residue. The absorbance was taken at 620 nm. A calibration curve was plotted by carrying out the same experiment using commercial retinol palmitate at varying concentrations in place of the extracts. The reagent mixture (in the absence extract) served as blank. The concentration of vitamin A present in AVHE and DAVHE were extrapolated from the retinol calibration curve and presented in milligrams per gram retinol equivalent.

8. Estimation of total vitamin E content

The total vitamin E content was assessed using the method described by Achikanu *et al*. (2013) with slight modifications. The assay is centred on the formation of a red complex between the reagent 2, 2-bipyridyl and Fe^{2+} which result from the oxidation of tocopherol with FeCl3. The vitamin E content of AVHE and DAVHE were determined as follows: alcoholic potassium hydroxide (2 mL, 0.5 N) was mixed with 0.05 g of each extract and the resulting mixtures were heated for 30 minutes in a water bath. Afterwards, 3 mL of n-hexane was pipetted into each mixture and vigorously shaken. The n-hexane was poured to another clean test tubes and evaporated to dryness. Ethanol (2 mL) was added to the residue, followed by 1 mL of 0.2% ferric chloride in ethanol. Afterwards, 1 mL of 0.5% (w/v) 2,2-bipyridyl in ethanol was added, after which 1 mL ethanol was added to make up the volume to 5 mL The assay mixtures were mixed vigorously and the absorbance were taken at 520 nm against a blank (reagent mixtures in the absence of extract). The vitamin E content of AVHE and DAVHE were deduced from tocopherol calibration curve and presented in milligrams per gram tocopherol equivalent.

9. Antioxidant assays

1) 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

The DPPH free radical scavenging activity of AVHE and DAVHE were evaluated using a modified version of the method described by Malterud & Ryland (2000). 1 mL of each extract at various concentrations (62.5, 125, 250, 500, and 1000 μg/mL) was combined with 1 mL of a freshly prepared DPPH methanol solution (0.004% (w/v)). The resulting mixtures were allowed to stand in the dark for 15 minutes at room temperature, after which the absorbance of each mixture was measured at 517 nm. Ascorbic acid was used as a reference standard for DPPH radical scavenging. The percentage scavenging activity of the extract was calculated using the following formula:

Percentage scavenging activity=[(A− B)/A)]×100

Where A=Absorbance of blank

B=Absorbance of sample

2) Ferric reducing antioxidant power assay

The reducing power of AVHE and DAVHE were determined using a method described by Chu (2000). Specifically, different concentrations of the extract (1 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and $K_4Fe(CN)_6$ (2.5 mL, 1% w/v). The assay mixtures were then incubated for 20 min at 50℃. Trichloroacetic acid, TCA (2.5 mL of 10% w/v) was added to each mixture, which was subsequently centrifuged for 10 minutes at 3000 rpm. The upper layer of the resulting solutions (2.5 mL) were collected and mixed with distilled water (2.5 mL) and iron (III) chloride, $FeCl_3$ (2.5 mL, 0.1% w/v). Ascorbic acid was used as the standard reference. The absorbance of each assay mixture was then measured at 700 nm using a spectrophotometer (S23A; Spectrumlab, England).

3) Lipid peroxidation inhibitory assay

The inhibition of lipid peroxidation by AVHE and DAVHE were investigated using a modified thiobarbituric acid reactive species (TBARS) reaction assay as reported by Gülçin *et al*. (2004). The reaction mixtures contained 0.5 mL of egg yolk aqueous homogenate 10% (w/v), varying concentrations of aqueous solution of extract prepared with 0.5% tween−80 as surfactant (50−250 μg/mL, 0.1 mL), and copper (II) tetraoxosulphate (VI) solution (0.05 mL; 70 mM). The mixtures were incubated for 30 min at 25℃, after which 20% acetic acid (1.5 mL; pH 3.5) and 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulfate (1.5 mL) was added. The assay mixtures were then heated for 1 hour at 95℃. After cooling, butanol (5.0 mL) was added and the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant's absorbance was later measured at 532 nm using a spectrophotometer (S23A; Spectrumlab, England). Ascorbic acid was used as the positive control in place of the extract. The percentage inhibition of lipid peroxidation was calculated with the formula below:

Percentage inhibition=$[(A_0 - A_1)/A_0]\times 100$

Where A_0=Absorbance of control

A_1=Absorbance of sample

4) Determination of total antioxidant capacity

The total antioxidant capacity of AVHE and DAVHE were estimated using the phosphomolybdenum method as described by Prieto *et al*. (1999). The assay was conducted as follows: extract (0.3 mL) was mixed with 3 mL of a reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The reacting mixtures were left to incubate for duration of 90 minutes in a water bath that was set to 95℃. After cooling to room temperature, absorbance at 695 nm was measured using a spectrophotometer (S23A; Spectrumlab, England) and compared to a blank control which contained distilled water instead of the extract. The antioxidant activities were presented as the number of grams of equivalent ascorbic acid.

5) Metal chelating assay

The metal chelating activity of AVHE and DAVHE was evaluated via the technique of Singh & Rajini (2004) with slight modifications. Aliquot (1 mL) of various concentrations of each extract (15.625−500 μg/mL) prepared with 0.5% tween−80 as surfactant, was mixed with $FeCl_2 \cdot 4H_2O$ and incubated for 5 minutes. Subsequently, ferrozine (1 mL) was added to initiate the reaction. The assay mixture was shaken vigorously and incubated for an additional 10 minutes. The absorbance of each reaction mixture was then measured at 562 nm using a UV−Visible spectrophotometer (110015; Labtronics Technologies Pvt. Ltd, India). The percentage inhibition of ferrozine−Fe^{2+} complex formation was calculated according to the following formula:

% Chelating effect=$[(A - B)/A]\times 100$

Where A=Absorbance of blank,

B=Absorbance of sample

10. Anti−inflammatory assays

1) Lipoxygenase inhibitory assay

The inhibitory activity of AVHE and DAVHE on lipoxygenase was investigated using a UV/visible light spectrophotometer

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method with linoleic acid as lipoxygenase substrate, based on the method described by Malterud & Rydland (2000) with some modifications. The assay mixture contained 150 μL phosphate borate buffer (2M, pH 9.0), 50 μL of extract (0.015 mg/mL-1.000 mg/mL), and 50 μL of the enzyme solution (167 U/mL in phosphate borate). The reaction was started by the addition of 250 μL of a substrate solution (0.15 mM). The kinetics of the enzymatic reaction was monitored for 5 minutes at 234 nm using a UV-Visible spectrophotometer (110015; Labtronics Technologies Pvt. Ltd, India). A negative control contained 1% methanol instead of the extract while various concentrations of quercetin (0.003-0.1 mg/mL) served as the standard lipoxygenase inhibitor. The percentage inhibition of lipoxygenase was calculated using the following formula:

$\%\ Inhibition=[Abs_{control}- Abs_{sample})/Abs_{control}]\times 100$

2) Trypsin inhibitory assay

The inhibitory activity of AVHE and DAVHE on trypsin was determined according to the modified method of Oyedapo & Famurewa (1995) as reported by Marrassini *et al*. (2018). The assay mixture (2 mL) contained 0.06 mg trypsin, 1 mL of Tris-HCl buffer (20 mM, pH 7.4), and 1 mL of the extract at several concentrations (100-500 μg/mL). The assay mixtures were incubated at 37℃ for 5 minutes, followed by the addition of casein (1 mL; 0.08% w/v). The assay mixtures were incubated further for 20 minutes, after which 1 mL of 70% (w/v) perchloric acid was added to terminate the reaction. The absorbance of the suspensions was measured at 280 nm against a buffer blank. An aqueous solution of Diclofenac was utilized as the positive control. The percentage of inhibition of trypsin activity was calculated with the formula.

$\%Inhibition=[(B/A)/A]\times 100$

Where A=Absorbance of blank

B=Absorbance of sample

3) Protein denaturation inhibitory assay

The inhibitory activity of AVHE and DAVHE on protein denaturation was evaluated using a method described by Mizushima & Kobayashi (1968) with slight modifications by Aina & Oyedapo (2013). The assay mixture contained albumin (0.5 mL, 1.5 mg/mL) and various concentrations of the extract (0.05-0.3 mg/mL). The mixture was incubated at 37℃ for 20 minutes and then heated at 57℃ for 3 minutes. Then, phosphate buffer (2.5 mL, 0.5 M, pH 6.3) was added to the mixture. The solution (1 mL) from each reaction mixture was pipetted into clean, dry test tubes in triplicates, after which Copper-Alkaline reagent (1 mL) and Folin-Ciocateu's Phenol reagent [1 mL; 10% (w/v)] were added. The reaction mixtures were incubated further at 55℃ for 10 minutes, cooled, and the absorbance was measured at 650 nm against a reagent blank using a spectrophotometer (S23A; Spectrumlab, England).

The quantity of protein left was calculated using the expression:

$[(Abs_{test}-Abs_{blank})/(Abs_{standard}-Abs_{blank})]\times 100$

The percentage inhibition was further calculated using the expression:

(Quantity of protein left/Total protein)×100

11. Anti-aging assays

1) Collagenase inhibitory assay

The collagenase inhibitory activity was determined using BioVision's Collagenase Activity Assay Kit by measuring the decrease of the substrate, a synthetic peptide, FALGPA (2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine), which mimics collagen's structure over time. Each extract (2 μL) at concentrations ranging from 2-10 mg/mL were incubated with 10 μL of collagenase for 10 minutes. A parallel well (control) containing 10 μL collagenase only. The volume of the test solution and the enzyme control was adjusted to 100 μL with collagenase assay buffer. Afterwards, FALGPA (40 μL) and collagenase assay buffer (60 μL) were pipetted into each reaction mixture. Absorbance of the mixtures was measured kinetically at 345 nm in a microplate reader at 37℃ for 15 minutes using UV-visible spectrophotometer (752D; Shimazu, Japan). The formula below was utilized to calculate the activity of collagenase enzyme:

$Collagenase\ activity=[(B-A)\ x\ (0.2)\times DF]/[(0.53)\times V]$

A=Absorbance of blank,

B=Absorbance of sample

DF=Dilution factor

0.53=millimolar extinction coefficient of FALGPA

0.2=Reaction volume (mL)

V=enzyme volume (mL)

The formula below was employed to calculate the degree of inhibition:

$\%Inhibition =[(Activity_{Enzyme}-Activity_{Inhibitor})/(Activity_{Enzyme})]\times 100$

2) Tyrosinase inhibitory assay

The inhibitory activities of AVHE and DAVHE on tyrosinase

were investigated using a protocol described by Ashraf *et al*. (2021) with slight modifications. Phosphate buffer (140 mL, 20 mM, pH 6.8), 20 mL tyrosinase from mushroom (40 U/mL), and 60 mL of the aqueous solution of extract (0.0625–1.000 mg/mL) prepared with 0.5% Tween–80 as surfactant, were pipetted into a 96–well microplate. After incubation at room temperature for 10 minutes, L–DOPA (3, 4–dihydroxyphenylalanine) (40 µL, 3 mM) was pipetted into the reaction mixture and the plate was incubated at 25℃ for a duration of 20 minutes. Absorbance of the resulting reaction mixture was then taken at 475 nm with a microplate reader using a UV–Visible spectrophotometer (110015; Labtronics Technologies Pvt. Ltd, New Delhi, India). Kojic acid served as a standard inhibitor, while phosphate buffer acted as a tyrosinase inhibitor control.

The formula below was used to calculate the percentage tyrosinase inhibitory activity

Tyrosinase activity (%)=[(A–B)/(C–D)]×100

Where A=absorbance of reaction mixture which contains sample and tyrosinase,

B=absorbance of blank sample containing sample without tyrosinase,

C=absorbance of reaction mixture with tyrosinase but without test sample

D=absorbance of the reaction mixture without test sample nor tyrosinase (L–DOPA alone).

12.Determination of photoprotective activity and UV blocking ability

The method described by Napagoda *et al*. (2016) was utilized to study the ability of the AVHE and DAVHE to filter UV light. The UV absorbance of the extract at concentrations of 1 mg/mL and 2.5 mg/mL was assessed over the range of 260 nm to 350 nm with a UV–visible spectrophotometer (110015; Labtronics Technologies Pvt. Ltd, New Delhi, India). Furthermore, the "Sun Protection Factor" (SPF) value of the extracts were computed using Mansur equation (Mansur *et al*. 1986).

$$SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where EE (λ) represents erythemal effect from 290 to 320 nm; I (λ) is the solar intensity from 290 to 320 nm; CF is the correction factor (10) and Abs (λ) is the absorbances of the extract or commercial sunscreen cream from 290 to 320 nm. The standardized product function EE×I at the wavelengths range of 290 to 320 nm were obtained from literature as reported by Majalekar *et al*. (2020).

13. Gas chromatography–mass spectroscopy analysis

The GC–MS analysis of AVHE was done using a Varian 3800/4000 gas chromatograph mass spectrometer (Agilent, USA) equipped with an Agilent fitted with a capillary column DB–5ms (30.0 m×0.25 mm, 0.25 µm film thickness). The GC column oven temperature was programmed from 70℃ (hold time 2 min) to 300℃ (hold time 7min) at the rate of 10℃ min–1with a total run time was 32.0 min. Nitrogen (99.9995% purity) was used as carrier gas at a flow rate of 1.51 mL/min. The GC–MS interface temperature was at 280℃. Injector and detector temperatures were set at 200℃. 1 µL of sample was injected in split ratio of 1:10. The MS scan range was set from 40–1000 Da.

The compounds obtained were identified by comparing the retention times with those of the standard compounds and with the spectral data obtained from data library using the database of National Institute Standard and Technology MS library (NIST–MS library).

14. Statistical analysis

The results were analyzed via one–way analysis of variance (ANOVA). The data, which were expressed as mean±SEM, were further analyzed using Graph Pad Prism 5 demo software, and the differences between the means were regarded statistically significant at $p<0.05$ (ANOVA).

Table 1. Chlorophyll, vitamin A, vitamin E, phenolics and flavonoid contents of AVHE and DAVHE

Extract	AVHE	DAVHE
Total chlorophyll (mg/mL)	19.37±0.09	11.90±0.07
Vitamin A (mgRE/g extract	87.29±0.84	41.29±0.74
Vitamin E (mgTE/g extract)	12.04±0.11	7.31±0.82
Total flavonoid (mgQE/g extract)	0.68±0.00	2.13±0.00
Total phenolic (mgGAE/g extract)	9.89±0.21	1.43±0.08

Each value signifies the mean±SEM, where n=3 replicates.
AVHE, non-dehlorophyllized *Asystasia vogeliana* leaf n-hexane extract; DAVHE, dechlorophyllized *Asystasia vogeliana* leaf n-hexane extract; RE, retinol equivalent; TE, tocopherol equivalent; QE, quercetin equivalent; GAE, gallic acid equivalent.

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Results

1. Chlorophyll, vitamin A, vitamin E, phenolics and flavonoid contents

The total chlorophyll, phenolic and flavonoid as well as vitamins A and E contents of AVHE and DAVHE are presented in Table 1. It was noted that the non-dechlorophyllized *A. vogeliana* leaf extract contains significantly higher level of vitamin A, vitamin E and total phenolic compounds than it dechlorophyllized counterpart ($p<0.05$).

2. Antioxidant activities of *A. vogeliana* leaf n- hexane extracts.

1) DPPH radical scavenging activity

The results of DPPH scavenging activity of *A. vogeliana* leaf n-hexane extracts showed that DAVHE possessed higher DPPH radical scavenging activity [0.918±0.016 mg/mL (IC_{50})] compared to AVHE, 1.197±0.034 mg/mL (IC_{50}) but not comparable to ascorbic acid standard [0.012±0.078 mg/mL (IC_{50})].

2) Lipid peroxidation inhibitory activity

The study demonstrates that AVHE {0.260±0.008 mg/mL (IC_{50})} and DAVHE {0.201±0.021 mg/mL (IC_{50}) provided protection against Fe^{2+}-induced lipid peroxidation in a concentration-dependent manner. The extracts were comparable with ascorbic acid {0.308±0.015 mg/mL (IC_{50})}, a known antioxidant, in their protective effects.

3) Metal chelating activity

The two extracts (AVHE and DAVHE) exhibited similar level of chelating activity (IC_{50}: 0.0762±2.533 mg/mL and 0.0771±1. 175 mg/mL) which was comparable EDTA (IC_{50}: 0.124±0.005 mg/mL).

4) Ferric reducing antioxidant power

The reductive power of AVHE (IC_{50}: 0.228±0.003 mg/mL) and DAVHE (IC_{50}: 0.322±0.013 mg/mL) shows that they possessed mild reductive activities compared to ascorbic acid (IC_{500}: 0.028±0.000 mg/mL).

5) Total antioxidant capacity

AVHE and DAVHE were found to possess substantial levels of antioxidant property as shown in their antioxidant capacities (0.922±0.001 mg/mL and 0.755±0.005 mg/mL) respectively.

3. Anti-inflammatory activities of *A. vogeliana* leaf n-hexane extracts

1) Trypsin inhibitory activity

The trypsin inhibitory activities of AVHE and DAVHE (Figure 1) were found to compare well with that of Diclofenac (a standard non-steroidal anti-inflammatory drug). The two extracts elicit similar inhibitory pattern at lower concentrations, however there was a significant (at $p<0.05$) increase in the inhibitory activity of DAVHE compared to AVHE at higher concentrations.

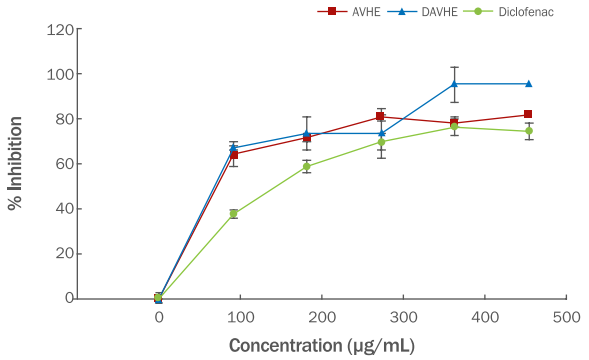

Figure 1. Trypsin inhibition of n-hexane extracts of *Asystasia vogeliana* leaf.
Each value represented the mean+SEM of n=3 replicates. AVHE, non-dehlorophyllized *Asystasia vogeliana* leaf n-hexane extract; DAVHE, dechlorophyllized *Asystasia vogeliana* leaf n-hexane extract.

2) Protein denaturation inhibitory activity

As depicted in Figure 2, AVHE and DAVHE demonstrated concentration-dependent inhibitory effect on the denaturation

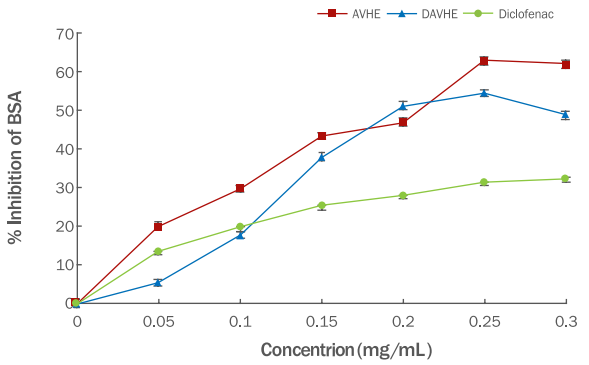

Figure 2. Effect of *Asystasia vogeliana* leaf n-hexane extract on bovine serum albumin denaturation.
Each value represented the mean+SEM of n=3 replicates. AVHE, *Asystasia vogeliana* n-hexane extract; DAVHE, dechlorophyllized *Asystasia vogeliana* n-hexane extract.

of bovine serum albumin (BSA). At all concentrations tested, the extracts displayed stronger protein denaturation inhibitory activity than Diclofenac. The maximum percentage inhibition of AVHE and DAVHE observed at 0.3 mg/mL were 62.07%±0.00 and 48.89%±0.02 respectively while Diclofenac exhibited a maximum percentage inhibition of 32.58%±0.02 at 0.3 mg/mL.

3) Lipoxygenase inhibitory activity

The study shows that AVHE (IC_{50}: 0.458±0.041 mg/mL) and DAVHE (IC_{50}: 0.190±0.000 mg/mL) possess mild lipoxygenase inhibitory activities compared to that of quercetin (IC_{50}: 0.0267±0.0001 mg/mL). However, it was observed that DAVHE possessed a higher level of lipoxygenase inhibitory activity than AVHE.

4. Anti-aging activities of *A. vogeliana* leaf n-hexane extracts

1) Anti-collagenase activity

It was observed that AVHE, DAVHE and Piroxicam, a standard collagenase inhibitor inhibited collagenase in concentration dependent manner (Figure 3), although, there is no significant difference in the collagenase inhibitory activities. The result showed that the dechlorophyllized and non-dechlorophyllized n-hexane *A. vogeliana* leaf extracts inhibited collagenase in the same manner but less comparable to Piroxicam.

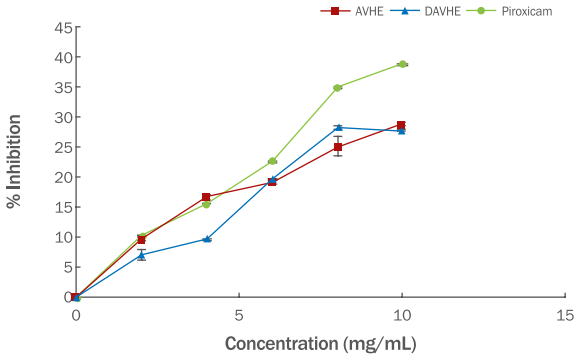

Figure 3. Inhibitory activities of piroxicam, AVHE and DAVHE against collagenase.
AVHE, *Asystasia vogeliana* n-hexane extract; DAVHE, dechlorophyllized *Asystasia vogeliana* n-hexane extract.

2) Anti-tyrosinase activity

It was recorded that AVHE (55.47±0.24% at 1 mg/mL) and DAVHE (54.62±3.52% at 1 mg/mL) possessed moderate tyrosinase inhibitory activity with similar mode of inhibition (Figure 4) while Kojic acid showed maximum inhibition of 80.81±4.86% at 1 mg/mL.

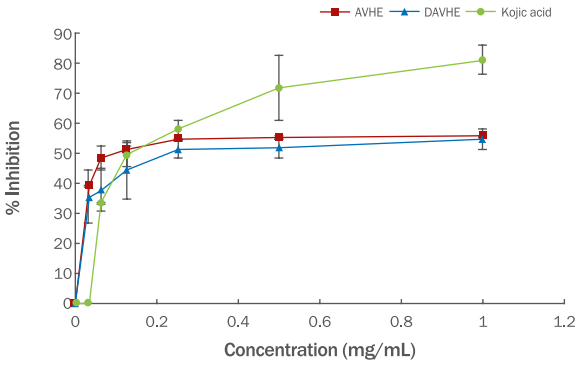

Figure 4. Tyrosinase inhibitory activities of kojic acid, AVHE and DAVHE.
AVHE, *Asystasia vogeliana* n-hexane extract; DAVHE, dechlorophyllized *Asystasia vogeliana* n-hexane extract.

5. Photoprotective activity and UV filtering potential

AVHE (SPF=46.35±0.55) and DAVHE (SPF=28.92±5.13) displayed SPF>25 at 2.5 mg/mL which were even greater than that of the commercial photoproprotective cream (SPF=11.65±0.01) used as standard reference. It was observed that the dechlorophyllized sample possess a lower SPF value than the non-dechlorophyllized sample. Interestingly, the real SPF value of the commercial sunscreen was found to be much lower than its labeled SPF value.

Moreover, AVHE and DAVHE (Figure 5) absorbed maximally at UV-C range (260-290 nm) while the maximum UV absorption of the standard commercial sunscreen lies within the UV-B range (290-310 nm).

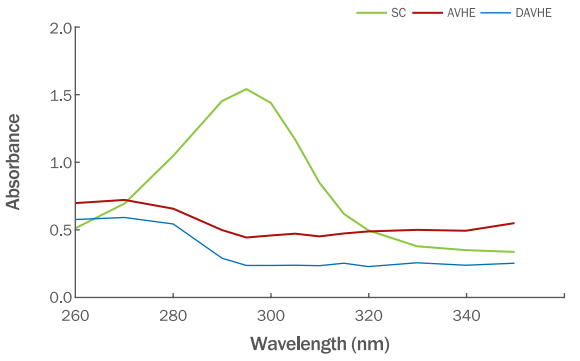

Figure 5. UV absorbance of AVHE, DAVHE and commercial sunscreen cream (SC).
AVHE, *Asystasia vogeliana* n-hexane extract; DAVHE, dechlorophyllized *Asystasia vogeliana* n-hexane extract.

6. GC-MS analysis

The chromatogram of the GC-MS analysis of AVHE is shown

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Table 2. Compounds identified in *Asystasia volgeliana* leaf n-hexane extract

Peak #	RT	Compound detected	Peak area %	Comp. wt%	m/z
1	11.61	Benzaldehyde	2.22	1.57	51, 77, 106
2	17.50	1-Piperazinecarboxaldehyde	3.88	2.73	44, 56, 114
3	18.50	Benzene, 1,2,3,5-tetramethyl-	5.65	2.91	41, 119, 134
4	18.54	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	1.94	2.02	43, 44, 144
5	20.60	1H-Pyrazole,3-methyl-5-phenyl-	4.43	1.96	42, 77, 158
6	21.00	2,4-Di-tert-butylphenol	4.16	5.71	41, 191, 206
7	21.68	1-Octadecyne	7.86	6.63	41, 81, 150
8	23.41	9-Octadecenoic acid	4.02	5.63	43, 73, 282
9	24.96	9,12,15-Octadecatrienoic acid, methyl ester	3.05	4.53	57, 79, 292
10	26.52	9,12-Octadecadienoic acid, methyl ester	6.20	7.38	41, 87, 294
11	28.00	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	6.70	5.11	42, 81, 296
12	28.97	n-Hexadecanoic acid	15.51	18.13	43, 73, 256
13	30.00	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	23.26	24.15	67, 79, 278
14	34.26	Phytol	6.79	7.39	71, 123, 296
15	35.50	Stigmasterol	2.77	2.91	55, 83, 412
16	37.96	Cycloheptasiloxane, tetradecamethyl-	1.33	1.06	73, 147, 519

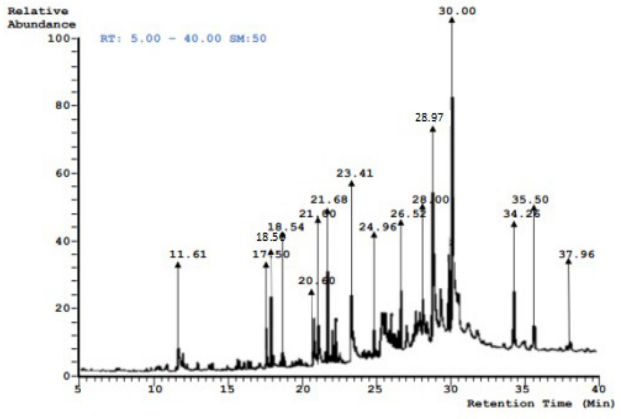

Figure 6. GC-MS chromatogram showing the mass spectra of AVHE.
AVHE, *Asystasia vogeliana* n-hexane extract.

in Figure 6. and the compounds identified from the resulting spectra are listed in Table 2. The major compounds identified in the extract are 9,12,15-Octadecatrienoic acid, (Z,Z,Z); n-Hexadecanoic acid; 1-Octadecyne and phytol.

Discussion

In the present study, the anti-skin aging potentials of dechlorophyllized (DAVHE) and non-dechlorophyllized (AVHE) samples of *A. vogeliana* n-hexane extracts were investigated. AVHE was recorded to possess higher levels of vitamin A, vitamin E and phenolic compounds than DAVHE which contained higher amount of total flavonoids. These bioactive compounds have been reported to function principally as strong antioxidants, which play crucial roles against various oxidative stress-related disorders including extrinsic skin aging. Oxidative stress is one of the most important processes which contribute to skin aging and several other dermatological disorders (Chaudhari *et al.*, 2011). Various plant species contain phenolic compounds, ascorbic acid, and carotenoids that have the ability to protect the skin (Petruk *et al.*, 2018). These plant substances can help to prevent UV radiation penetration into the skin, reduce inflammation and oxidative stress, and affect various signaling pathways that are essential for survival. More so, vitamins have been generally reported to confer protection against several disorders. A number of studies have shown that vitamin E (tocopherol) and vitamin A prevents various oxidative stress conditions. Topical application of tocopherol has been reported to impede lipid peroxidation and suppresses UV-B induced skin damage (Vuleta & Savić, 2009; Fisher *et al.*, 2000).

The reduction in the levels of phenolics and flavonoid contents, vitamin A and E of DAVHE could be as a result of the fact that some of these compounds co-extracted with the chlorophyll in acetone, this is in agreement with the study of Benjakul *et al.* (2014) that prior removal of chlorophyll reduced the phenolics in lead seed extract. More so, Laily *et al.* (2015) reported that

methanol, ethyl acetate, ethanol, water and acetone could be used to extract phenolic compounds.

The present findings revealed that AVHE and DAVHE possess antioxidant property as evaluated through the assays (DPPH scavenging, lipid peroxidation inhibition, metal chelation, ferric reducing power and total antioxidant capacity); this could be attributed to the existence of a number of secondary metabolites in the leaf as the bioactivity of most medicinal plants have generally been ascribed to the existence of secondary metabolites (Akinpelu *et al*., 2018).While numerical differences were observed in the IC_{50} of the two extracts, these differences were not statistically significant, indicating comparable antioxidant activity and the potential of *A. vogeliana* in combating oxidative stress, inflammation and age-related condition. This agrees with the report of Ugwuanyi *et al*. (2020) who investigated the in vitro antioxidant potentials of *A. vogeliana* leaf petroleum ether and methanolic extracts where it was observed that both extracts elicited antioxidant activities.

Over-exposure of skin to UV radiation can lead to an upsurge in the production of reactive oxygen species (ROS), which can then trigger the activation of both inflammatory and pro-inflammatory cytokines (Davinelli *et al*., 2018; Imokawa, 2018). Hence, anti-inflammatory property is one of the desirable features of plants extracts of dermatological importance. Protease inhibitors (including trypsin inhibitors) are short chains of amino acids with the ability to impede proteolytic biocatalysts such as trypsin and collagenase. Trypsin inhibitors display potent anti-inflammatory property, indicating their potential benefits in ameliorating inflammatory related conditions which include extrinsic skin aging (Shamsi *et al*., 2017). AVHE and DAVHE showed high protein denaturation and trypsin inhibition properties comparable to Diclofenac (non-steroidal anti-inflammatory drug) at all tested concentrations, suggesting its suitability in management of inflammatory related skin disorders. The study showed that DAVHE possessed a higher level of lipoxygenase inhibitory properties, however, both exhibit similar pattern of proteinase (trypsin) inhibition. Lipoxygenase catalyzes the reaction in which arachidonic acid is converted to pro-inflammatory leukotrienes, which are important inflammatory mediators (Schneider & Bucar, 2005).

Collagenase, an enzyme that degrades collagen and elastin, has been shown to contribute to early skin damage or wrinkle formation by breaking the amino acid bonds of collagen (Alipour *et al*., 2016). Madhan *et al*. (2007) proposed that the collagenase inhibition properties of certain compounds may be due to hydrophobic interactions between the benzene ring of polyphenols and collagenase, which could affect the enzyme's function. Additionally, the hydroxyl group of polyphenols present in an extract may react with the backbone or other functional groups in the side chains of collagenase. Dušan & Vesna (2007) suggested that the flavonoids present in an extract may chelate the Zn^{2+} present in the active site of collagenase, thereby preventing the breakdown of collagen fibers by collagenase and maintaining the integrity of the skin layer. The two extracts of *A. vogeliana* leaf investigated showed similar levels of collagenase inhibition with Piroxicam, suggesting that they are capable of delaying collagen breakdown, hence, maintaining skin integrity.

Tyrosinase catalyzes the first two steps in the formation of melanin from tyrosine, hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and oxidation of L-DOPA to dopaquinone which is eventually converted to melanin (Seo *et al*., 2003). Melanin protects the skin against UV radiation; however, its excessive formation in the skin can cause hyperpigmentation disorders, hence, an effective natural substance which inhibits tyrosinase is desirable in formulation of skin care products. This study showed that both the dechlorophyllized and non-dechlorophyllized n- hexane extracts of *A. vogeliana* leaf possess a moderate and similar level of tyrosinase inhibition (54.62%± 3.52 and 55.47 %±0.24 respectively at 1 mg/mL) in comparison with Kojic acid, a standard tyrosinase inhibitor (80.81% ± 4.86 at 1 mg/mL). Previous investigations suggested that the possible mechanism of the tyrosinase inhibitory activities of the plant extracts may involve the chelation of the copper atoms present in the active site of the enzyme, which contribute to the catalytic effect of the enzyme (Nithitanakool *et al*., 2009).

Sun Protection Factor (SPF) is a measure used in sunscreens formulation to indicate the level of the cream's protection against UV radiation when applied at a thickness of 2 mg/cm^2 of skin (Jangde & Daharwal, 2011; Trautinger, 2001). Sunscreen products can be classified based on their SPF values as minimal (SPF<12), moderate (SPF 12-30), and high (SPF≥ 30) (Stevanato *et al*., 2014). The level of protection offered by a sunscreen formulation against UV radiation varies based on its SPF value (Napagoda *et al*, 2016). Commercial sunscreen creams typically contain a combination of chemical filters, UV filters and/or moisturiers. These include titanium dioxide, zinc oxide, avobenzone, octocrylene amongst others (Matta *et al*., 2019). In the present investigation, AVHE was found to possess

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a high SPF value (>30) at 2.5 mg/mL suggesting its possible use as a sunscreen agent while the DAVHE possess moderate SPF value; interestingly, the standard sunscreen cream exhibits a low SPF value, contrary to its labeled value which is consistent with some previous researches indicating that the labeled SPF value may not be the actual SPF value (Fonseca & Rafaela, 2013). Many UV-blocking formulations only provide protection within a specific range of the UV spectrum (Napagoda *et al*., 2016), this agrees with the result of the present investigation as the commercial sunscreen was found to possess high UV absorption in the UV B region (Figure 6), however, AVHE and DAVHE elicits their highest UV absorbance in the UV C region. Although, the presence of the ozone layer is expected to effectively block UV C radiation from the sunlight (Bias *et al*., 2015), however, the ozone layer is gradually being depleted thereby allowing the penetration of UV C radiation which may be extremely harmful to the skin. The high UV blocking potential of *A. vogeliana* n-hexane leaf extracts in the UV C region suggests a promising use in the formulation of UV-C blocking sunscreens. The UV blocking potential could be as a result of the presence of various phytochemicals which are capable of absorbing UV light at different range of wavelengths. Chlorophyll and its derivatives may contribute to the overall photoprotective properties of AVHE due to their ability to absorb and neutralize free radicals generated by UV exposure (Kotkowiak *et al*., 2017). Conversely, the removal of chlorophyll and potentially other co-extracted compounds in DAVHE might have led to a reduction in SPF values. However, there is need for further investigations to validate the role of chlorophyll in SPF efficacy.

The GC-MS profile of AVHE showed that it contains an array of diverse biologically active compounds. AVHE was shown to be rich in diverse polyunsaturated fatty acids (PUFAs) and other bioactive compounds including 9,12,15-Octadecatrienoic acid, (Z,Z,Z) (23.26%); n-Hexadecanoic acid (15.51%); 1-Octadecyne (7.86%) and phytol (6.79%). Polyunsaturated fatty acids have been reported to be crucial in maintaining the integrity of human skin, and possess anti-inflammatory properties (Bali´c *et al*., 2020). Phytol had been shown to possess anti-inflammatory, anti-microbial, antioxidant and tyrosinase inhibitory properties (Ko & Cho, 2018). While antioxidant as well as antibacterial activities of 9,12,15-Octadecatrienoic acid, (Z,Z,Z) have been documented (Godwin *et al*., 2015).

Conclusion

Asystasia vogeliana leaf n-hexane dechlorophyllized and non-dechlorophyllized extracts possess antioxidant, anti-inflammatory properties, photoprotective and moderate collagenase as well as tyrosinase inhibitory activities suggesting their promising skin aging retardation properties. However, prior removal of chlorophyll using an aprotic solvent (acetone) has a slight reducing impact on these properties. These findings suggest that the extract has the potential to be used in the development of effective herbal cosmetics; however, better means of chlorophyll removal may be employed. Also, more detailed in vivo and cell-based studies should be carried out to validate the suitability of *Asystasia vogeliana* leaf extract in the development of herbal cosmetics.

Author's contribution

Makinde B. I., Godwin A. and Akinpelu B. A. each made significant contributions to this work. Akinpelu B. A. and Makinde B. I. designed the experimental investigations. Makinde B. I. carried out the experiments under the supervision of Akinpelu B. A. Godwin A. contributed to the experimental design and data analysis. Makinde B. I. wrote the manuscript with assistance from Akinpelu B. A.

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국문초록

Asystasia vogeliana 잎 n-헥산 추출물 및 그 탈염소화 등가물의 항산화, 항염증 및 피부 노화 효소억제 작용

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목적: 본 연구에서는 *Asystasia vogeliana* 잎 n-헥산 추출물(AVHE)과 그 탈엽록소 추출물(DAVHE)의 항산화, 항염증, 광보호 및 피부 노화 지연 활성을 조사하였다. **방법:** 추출물의 총 페놀릭, 플라보노이드, 비타민 A 및 비타민 E 함량을 정량화하였으며, 항산화(DPPH 소거, 철 환원 항산화력, 총 항산화능, 지질 과산화 및 금속 킬레이트화 억제), 항염증(리폭시게나제, 트립신 및 단백질 변성 억제), 광보호(SPF 및 UV 차단) 및 피부노화 효소(콜라게나제 및 티로시나제 억제) 등은 표준 방법을 사용하여 평가하였다. 추출물의 활성 성분은 GC-MS를 통해 확인하였다. **결과:** AVHE와 DAVHE는 약간의 총 페놀성 물질(9.89±0.21 mgGAE/g 및 1.43±0.08 mgGAE/g)과 플라보노이드(0.68±0.00 mgGAE/g, 2.13±0.00 mgGAE/g)를 각각 함유하고 있습니다. AVHE (87.29±0.84 mgRE/g, 12.04±0.11 mgTE/g)는 DAVHE (41.29±0.74 mgRE/g, 7.31±0.82 mg TE/g)보다 비타민 A 및 E 함량이 유의하게 더 높았다. 두 추출물 모두 적당한 항산화 활성을 나타냈다. AVHE는 더 높은 트립신 억제(81.44%±0.83), 단백질 변성 억제(62.07%±0.00) 및 리폭시게나제 억제(IC_{50}=0.458±0.041 mg/mL)를 나타냈다. 두 추출물 모두 유사한 콜라게나제 및 티로시나제 억제 활성을 나타냈다. AVHE는 DAVHE (28.92±5.13) 및 상업용 자외선 차단제(11.65±0.01)보다 훨씬 더 높은 광보호 활성(46.35±0.55)을 나타냈다. GC-MS 분석을 통해 AVHE에 9,12,15-옥타데카트리엔산(Z,Z,Z); n-헥사데칸산; 1-옥타데신과 피톨 등 주요한 성분을 확인하였다. **결론:** *A. vogeliana* 잎 n-헥산 추출물은 스킨 케어제의 유망한 후보물질이며 탈엽록소화는 그 효능을 약간 감소시킨다.

핵심어: *Asystasia vogeliana*, 항산화, 항염증, 피부노화효소, 광보호

中文摘要

Asystasia vogeliana 叶正己烷提取物及其脱叶绿素等效物的抗氧化、抗炎和皮肤老化酶抑制作用

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目的：该研究调查了*Asystasia vogeliana*叶正己烷提取物(AVHE)及其脱叶绿素等效物(DAVHE)的抗氧化、抗炎、光保护和延缓皮肤衰老活性。**方法：**对提取物中的总酚类、类黄酮、维生素A和维生素E含量进行定量。使用标准方法测定抗氧化(DPPH清除、铁还原抗氧化能力、总抗氧化能力、脂质过氧化和金属螯合抑制)、抗炎(脂氧合酶、胰蛋白酶和蛋白质变性抑制)、光保护(SPF和紫外线阻挡)和皮肤老化酶(胶原酶和酪氨酸酶抑制)等活性。通过GC-MS技术鉴定了最有效的提取物的成分。**结果：**AVHE和DAVHE含有少量总酚(9.89±0.21 mgGAE/g和 1.43±0.08 mgGAE/g)和类黄酮(0.68±0.00 mgGAE/g、2.13±0.00 mgGAE/g)；AVHE的维生素A和E含量(87.29±0.84 mgRE/g、12.04±0.11 mgTE/g)显着高于DAVHE(41.29±0.74 mgRE/g、7.31±0.82 mgTE/g)。两种提取物均显示出中等的抗氧化活性。AVHE表现出较高的胰蛋白酶抑制作用(81.44%±0.83)、蛋白质变性抑制作用(62.07%±0.00)和脂氧合酶抑制作用(IC_{50}=0.458±0.041 mg/mL)。两种提取物具有相似的胶原酶和酪氨酸酶抑制活性。AVHE的防晒活性(46.35±0.55)明显高于DAVHE(28.92±5.13)和商业防晒霜(11.65±0.01)。GC-MS分析确定了AVHE中的主要成分，包括9,12,15-十八碳三烯酸(Z,Z,Z)，正十六烷酸以及1-十八炔和植醇。**结论：***A. vogeliana* 叶正己烷提取物是皮肤护理配方的有前途的候选者。然而，脱叶绿素作用会稍微降低其功效。

关键词：*Asystasia vogeliana*，抗氧化，抗炎，皮肤老化酶，光保护

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