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ORIGINAL ARTICLE

Evaluation of Skin Anti-aging Potential of *Citrus reticulata* Blanco Peel

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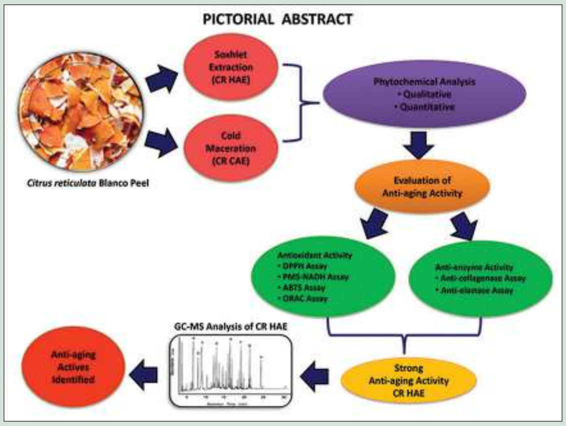
ABSTRACT

Background: The peel of *Citrus reticulata* Blanco is traditionally used as tonic, stomachic, astringent, and carminative. It is also useful in skin care. **Objective:** To study the anti-aging potential of alcoholic extracts of *C. reticulata* Blanco peel using *in vitro* antioxidant and anti-enzyme assays. **Materials and Methods:** Plant extracts were obtained by Soxhlation (CR HAE- Hot Alcoholic Extract of *Citrus reticulata*) and maceration method (CR CAE- Cold Alcoholic Extract of *Citrus reticulata*). Qualitative and quantitative phytochemical analysis was performed. Further, *in vitro* antioxidant, anti-enzyme, and gas chromatography-mass spectrometry (GC-MS) analyses were performed. **Results:** Total phenolic and flavonoid contents of CR HAE were found to be higher than CR CAE. EC_{50} value of CR HAE and CR CAE for 1,1-Diphenyl-2-picrylhydrazyl, Superoxide anion, and 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assays were 250.33 ± 40.16 μg/ml and 254.73 ± 15.78 μg/ml, 221.27 ± 11.25 μg/ml and 354.20 ± 23.79 μg/ml, and 59.16 ± 2.17 μg/ml and 59.12 ± 6.21 μg/ml, respectively. Oxygen radical absorbance capacity values for CR HAE and CR CAE were found to be 1243 and 1063 μmoles 6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid equivalent/g of substance, respectively. Anti-collagenase and anti-elastase activities were evaluated for both CR HAE and CR CAE. EC_{50} values of CR HAE and CR CAE for anti-collagenase and anti-elastase were 329.33 ± 6.38 μg/ml, 466.93 ± 8.04 μg/ml and 3.22 ± 0.24 mg/ml, 5.09 ± 0.30 mg/ml, respectively. CR HAE exhibited stronger anti-collagenase and anti-elastase activity than CR CAE. GC-MS analysis of CR HAE was carried out because CR HAE exhibited higher antioxidant and anti-enzyme potential than CR CAE. **Conclusion:** *C. reticulata* peel can be utilized in anti-wrinkle skin care formulations.

Key words: 1,1-Diphenyl-2-picrylhydrazyl, 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), anti-wrinkle, elastase, gas chromatography-mass spectrometry, oxygen radical absorbance capacity

SUMMARY

- Skin anti-aging potential of *Citrus reticulata* Blanco peel was evaluated through
- *In vitro* antioxidant and anti-enzyme assays
- Two types of extraction were performed and extracts were subjected to qualitative and quantitative phytochemical analysis. Extract obtained by Soxhlation (CR HAE) showed higher total phenolic and flavonoid contents than extract obtained by maceration (CR CAE)
- CR HAE demonstrated strong DPPH and Superoxide free radical scavenging activity whereas, ABTS scavenging activity of both the extracts were found to be similar. Oxygen Radical Absorbance Capacity (ORAC) of CR HAE was found to be more; indicating its strong antioxidant potential
- *In vitro* collagenase and elastase enzyme inhibition activities were evaluated for both the extracts and CR HAE showed strong anti-collagenase and anti-elastase potential indicating its anti-aging ability
- GC-MS analysis of CR HAE revealed the presence of various compounds mainly including Polymethoxyflavones. CR HAE exhibited promising antioxidant and anti-enzymatic activity and can be used as a potent anti-wrinkle agent in anti-aging skin care formulations.



Abbreviation Used: ECM: Extracellular matrix, UV: Ultra violet, ROS: Reactive Oxygen Species, MMP: Matrix metalloproteinase, Chc: *Clostridium histolyticum* collagenase, DPPH: 2, 2-diphenyl-1-picrylhydrazyl, GC-MS: Gas Chromatography- Mass Spectroscopy, RT: Room Temperature, μg GAE/ mg: Microgram Gallic acid equivalent / milligram, W/V: Weight by Volume, μg QE/ mg: Microgram Quercetin equivalent / milligram, CR HAE: Hot Alcoholic Extract of *Citrus reticulata* Blanco, CR CAE: Cold Alcoholic Extract of *Citrus reticulata* Blanco, EC50: Half Maximal Effective Concentration, PMS NADH: Phenazine methosulfate nicotinamide adenine dinucleotide, NBT: Nitroblue tetrazolium, DMSO: Dimethyl sulfoxide, APS: Ammonium Persulphate, AAPH: 2,2 -azobis(2-amidino-propane) dihydrochloride, TROLOX: (±) 6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid, ORAC: Oxygen Radical Absorbance Capacity, FALGPA: N-[3-(2-Furyl) acryloyl)]-Leu-Gly-Pro-Ala, SANA: Succinyl-Ala-Ala-Ala-p-nitroanilide, Rf: Retardation Factor, MSD: Mass Selective Detector

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INTRODUCTION

The most important role of skin for human being is to create a barrier between inside and outside environment of the body. Internally, the skin shelters and protects the entire physiochemical phenomenon necessary for life, externally it is a barrier against mechanical forces. Skin is the largest organ of the body.[1] It is a very sensitive organ and can easily get damage by infection and diseases.

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Skin problems can arise due to various factors such as environmental pollution, over exposure to ultraviolet rays, age of individual, harmful microorganisms, eating habits, stress, and chemicals. Skin treatment and care is essential not only to have a healthy skin, but also for the overall well-being of the person.[2]

One of the major skin problems in today's world is premature skin aging. The process of skin aging has been divided into two categories: Intrinsic and extrinsic aging. Intrinsic skin aging or natural aging is caused by changes in elasticity of the skin over time. Extrinsic skin aging is predominately a result of exposure to solar radiation (photo aging). Free radicals formed due to oxidative stress, play a major role in the course of both intrinsic and extrinsic aging.[3] Skin is made up of three main layers; epidermis, dermis, and subcutis. The major components of dermis are collagen and elastin fibers. Reduction in collagen and elastin leads to the wrinkle formation.[4] The main reason that skin starts to sag and form wrinkles is the breakdown in elastin and collagen. Collagenase and elastase enzymes are responsible for breakdown of various components of the extracellular matrix (ECM), i.e., collagen and elastin. Elastin is a protein that helps the skin stay supple and firm. When your skin is stretched, it is the elastin that returns it to its normal position. Collagen is a fibrous protein. Collagen is special among proteins because of its great tensile strength providing firmness to the skin. Wrinkles result from a combination of intrinsic and extrinsic aging as well as due increased level of collagenase and elastase enzymes.[5]

Cosmetics are used regularly and universally in different forms to enhance beauty. Skin care cosmetics treat the surface layer of the skin by providing better protection against the environment than skin left untreated.[6] There is an increasing demand for facial skin care cosmetics. According to data monitor, global spending on skin care products in 2012 was 82 billion $, where two-thirds of spending comprised facial skin care. A report by research and markets, expects the global skin care products industry revenue to cross 100 billion $ in 2018. Facial care segment is expected to continue to dominate the market. The increasing demand for anti-aging products and growing concern for the use of natural and organic skin care products are the major factors driving the skin care industry.[7]

Cosmetics alone are not sufficient to take care of skin and body parts; it requires association of active ingredients to check the damage and aging of the skin. These herbal actives which contain active ingredients are very essential to maintain the skin health. Cosmetics with herbal actives are now emerging as an appropriate solution to the current problem.[8]

In the present study, skin anti-aging potential of alcoholic extracts of *Citrus reticulata* Blanco peel were evaluated by *in vitro* antioxidant, anti-collagenase, and anti-elastase assays. *C. reticulata* Blanco (*Rutaceae*) is commonly known as Narangi or Santra (Orange). The fruit is laxative, aphrodisiac, astringent, tonic, and relieves vomiting.[9] The fruit peel is traditionally used as tonic, stomachic, astringent, carminative, and anti-scorbutic. Citrus peel has been regarded as a by-product of the citrus fruit industry, but it has been reported to contain several active components at far higher concentration than the pulp.[10] The fruit peel is also useful in skin care.

MATERIALS AND METHODS

Chemicals and reagents

Ascorbic acid, quercetin, ferric chloride, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), aluminum chloride ($AlCl_3$), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), porcine pancreatic elastase (EC.3.4.21.36), Succinyl-Ala-Ala-Ala-p-nitroanilide (SANA), *Clostridium histolyticum* collagenase (ChC) (EC.3.4.23.3), N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA), fluorescein sodium salt, 2,2-azobis (2-methyl propionamidine) dihydrochloride (AAPH), and gallic acid were purchased from Sigma Chemical Co. (St. Louis, USA.) 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±) 6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid (TROLOX) acquired from Fluka, USA. Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), and nicotinamide adenine dinucleotide - reduced disodium salt (NADH) were obtained from SRL Pvt. Ltd., Mumbai, India. Ammonium per sulfate (APS) was purchased from Rankem, India.

Plant material collection

Fruits of *C. reticulata* Blanco, *Rutaceae* were obtained in January 2013 from Mumbai (India). The taxonomic identification of plant material was confirmed by Dr. C. S. Latto, University of Mumbai. Authentication was done at Agharkar Research Institute, Pune.

Extraction of plant material

Fruits were washed thoroughly under water. Peels were separated from fruits and dried under shade. Then, the dried material was subjected to the powder form and stored in an airtight container at room temperature. About 50 g of dried powder was extracted with 300 ml methanol. Plant extracts were obtained by Soxhlation (CR HAE- Hot Alcoholic Extract of *Citrus reticulata*) and maceration method (CR CAE- Cold Alcoholic Extract of *Citrus reticulata*). Soxhlet extraction was carried out at 65°C for 10–12 h. Cold maceration was carried out by constant shaking; where the mixture of plant powder and methanol was kept on a rotary shaker (REMI CIS-24 PLUS) and was set at 100 rpm at RT for 72 h. The extracts were filtered using Whatman filter paper no. 1, and the filtrates were then evaporated under reduced pressure and dried using a rotary evaporator (R-205, Buchi Laboratory Equipment, Flawil, Switzerland) set at 50°C. Dried extracts were stored at 4°C, in labeled, sterile, capped bottles until further use.

Preliminary phytochemical analysis of extracts

The extracts as mentioned above were subjected to various qualitative phytochemical tests for identification of chemical constituents present in the plant material according to the described methods.[11]

Determination of total phenolic content

Total phenolic content (TPC) were determined with the Folin - Ciocalteu reagent using the given method.[12] To 0.5 ml of each sample, 2.5 ml 1:10 dilution of Folin - Ciocalteau's reagent and 2 ml of Na_2CO_3 (7.5% w/v) were added and incubated at 765 nm using a ultraviolet-visible spectrophotometer. Results were expressed as μg of gallic acid equivalent per mg of extract (μg GAE/mg extract).

Determination of total flavonoid content

Total flavonoid content (TFC) was measured by the $AlCl_3$ colorimetric assay.[13]

An aliquot (1 ml) of extracts or standard solutions of quercetin (20, 40, 60, 80, and 100 μg/ml) was added to a 10 ml volumetric flask containing 4 ml of distilled water. To the flask, 0.30 ml of 5% $NaNO_2$ was added and after 5 min, 0.3 ml of 10% $AlCl_3$ was added. After 5 min, 2 ml of 1M NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm. The TFC was expressed as μg of quercetin equivalent per mg of extract (μg QE/mg extract).

Antioxidant assays

1, 1-Diphenyl-2-picrylhydrazyl free radical scavenging assay

The free radical scavenging activity was evaluated by given method with some modifications.[14] The free radical scavenging activity was evaluated by given method with some modifications. A volume of 1 ml of the different concentrations (100–800 μg/ml) of test samples was mixed with 200 μl of (0.36 mg/ml) DPPH methanol solution. After shaking, the mixture was incubated in dark at room temperature for 30 min, and then the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control.

Phenazine methosulfate-NADH system superoxide anion scavenging assay

The superoxide scavenging activity was evaluated by given method.[15] Tris HCl buffer (3 ml, 16 mM, pH 8.0) containing 0.75 ml NBT (50 μM) solution, 0.75 ml NADH (78 μM) solution, and 0.3 ml sample solution of extract (50–350 μg/ml) in methanol were mixed. The reaction was started when 0.75 ml of PMS solution (10 μM) was added to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance was read at 560 nm against the corresponding blank samples. Ascorbic acid was used as a standard.

2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

The assay is performed as per the given method.[16] ABTS radical cations were produced by reacting ABTS and APS and incubating the mixture at room temperature in dark for 16 h. In brief, the total reaction volume contained 10 mM PBS pH 7.4 (positive control or test solutions) of various concentrations. ABTS radical solution was added to a final concentration of 0.219 mM. The reaction mixture was mixed and immediately read at 734 nm using microplate reader (VERSA max, Molecular devices, USA). A control reaction was carried out without the test sample. Gallic acid was used as a positive control.

The ability of the extracts to scavenge DPPH, PMS-NADH radicals, and ABTS free radical was calculated by the following formula:

Radical scavenging activity (%) = ([Abs of control – Abs of sample]/Abs of control) ×100, where Abs is the absorbance at mentioned wavelength in the presence or absence of the test extracts.

Oxygen radical absorbance capacity assay

This assay was performed as per the method described.[17] A preincubation mixture of 140 μl contained – 20 μl of test solution or TROLOX of various concentrations. 75 mM sodium phosphate buffer, pH 7.4; 120 μl of sodium fluorescein (117 nM) mixed and incubated at 37°C for 10 min. Following preincubation, 60 μl of AAPH (40 mM) was added and mixed for 15 s. The reaction was carried out for 90 min at 37°C. The fluorescence measurements were taken at 485 nm excitation and 520 nm emission filters.

Enzyme assays

Anti-collagenase assay

Collagenase inhibition assay was performed by the method described previously,[18] which is based on the hydrolysis of FALGPA by collagenase to produce FA-Leu and Gly-Pro-Ala. The assay was performed in 50 mM Tricine buffer (400 mM NaCl and 10 mM $CaCl_2$, pH 7.5). ChC was dissolved in the buffer for use at an initial concentration of 0.8 units/ml. The synthetic substrate, FALGPA, was dissolved in the Tricine buffer to 2 mM. Sample extracts were incubated with the enzyme in the buffer for 15 min before adding substrate to start reaction. The final reaction mixture (75 μl total volume) contained 25 μl of 50 mM Tricine buffer, 25 μl of test extract (250–4000 μg/ml), and 25 μl of 0.1 units of enzyme ChC. Controls performed with 50 mM Tricine buffer as test extracts were dissolved in Tricine buffer (50 mM), while catechin was used as a positive control. After adding 50 μl of 2 mM FALGPA substrate, collagenase activity was measured immediately at 340 nm for 20 min using a 96 well micro plate reader (Bio-Tek M Quant, FLX 800).

Anti-elastase assay

The assay employed was based on methods from the literature.[19] This assay was performed in 0.2 mM Tris-HCL buffer (pH 8.0). Porcine pancreatic elastase (PE – E.C. 3.4.21.36) was dissolved to make a 1 mg/ml stock solution in 0.2 mM Tris-HCL buffer. The substrate N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (SANA) was dissolved in buffer at 0.8 mM. The test extracts (0.156–10 mg/ml) were incubated with the enzyme for 20 min before adding substrate to begin the reaction. The final reaction mixture (Total 250 μl) contained 50 μl plant extract, 160 μl buffer, 20 μl enzyme, and 20 μl substrate. Catechin was used as a positive control. Negative controls were performed using Tris-HCL buffer. Absorbance was measured immediately at 410 nm and then continuously for 20 min using a 96 well micro plate reader (Bio-Tek M Quant, FLX 800).

The percentage inhibition for both of these assays is calculated by:

Enzyme inhibition activity (%) = (1– [B/A]) ×100

Where, A = Enzyme activity without sample, B = Activity in the presence of sample.

Gas chromatography-mass spectrometry analysis

Following conditions were adopted for gas chromatography-mass spectrometry (GC-MS) analysis.[20] The component identification was done using Agilent 7890 A gas chromatograph coupled with 5975 C inert Mass Selective Detector (MSD) with Triple Axis Detector. Samples were injected by Agilent 7693 auto sampler into Agilent 19091S-433: HP-5MS column (30 m × 250 μm × 0.25 μm). Helium was used as a carrier gas (1 ml/min). Injector and detector temperature were kept at 250°C. Column temperature was programed at 60°C for 3 min and then raised up to 160°C for 2 min at 10°C/min. Further temperature was raised up to 300°C (at 15°C/min). Mass spectra were acquired over 40–500 amu range in EI mode. The eluted compounds were identified by comparing mass spectral data with the standard data available in the library of National Institute of Standards and Technology. Main constituents from Soxhlet methanolic extract of *C. reticulata* Blanco peel (CR HAE) were identified by GC-MS analysis.

Statistical analysis

The EC_{50} values were expressed as mean ± standard deviation, statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple comparison test ($P < 0.05$) where, all samples, i.e., test extracts and positive standard were compared with each other. All calculations were performed using GraphPad Prism (version 5.0, GraphPad Software, USA).

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of extracts

Preliminary phytochemical analysis of methanolic extracts of *C. reticulata* Blanco indicates the presence of carbohydrates, amino acids, flavonoids, tannins and phenolic derivatives, steroids, etc., [Table 1].

Table 1: Preliminary phytochemical analysis of methanolic extracts of *Citrus reticulata* Blanco peel

Test performed	Name of the test	CR HAE	CR CAE
Test for carbohydrates	Fehling's test	+	+
Test for amino acids	Ninhydrin test	+	+
Test for flavonoids	Shinoda test	+	+
Test for alkaloids	Dragendorff	–	–
	Mayer's	–	–
	Wagner's reagent	–	–
Test for tannins and phenolic compound	5% $FeCl_3$	+	+
Test for steroids	Salkowski reaction	+	+

$FeCl_3$: Ferric chloride; CR HAE: Hot Alcoholic Extract of *Citrus reticulata* Blanco; CR CAE: Cold Alcoholic Extract of *Citrus reticulata* Blanco

Determination of total phenolic content

The phenolics, particularly polyphenols exhibit a wide variety of beneficial biological activities in mammals, including antiviral, antibacterial, immune-stimulating, anti-allergic, anti-hypertensive, anti-ischemic, anti-arrhythmic, anti-thrombotic, hypocholesterolemic, anti-lipoperoxidant, hepatoprotective, anti-inflammatory, and anti-carcinogenic actions.[21,22] Several studies have shown the flavonoids to act as scavengers of superoxide anions, singlet oxygen, hydroxyl radicals, and lipid peroxyl radicals.[23,24] TPC and TFC were determined for both the extracts using standard curves [Figure 1]. CR HAE showed higher phenolic content, i.e., 187.93 ± 4.69 µg GAE/mg extract than CR CAE, i.e. 58.66 ± 2.40 µg GAE/mg extract. TFCs for CR HAE and CR CAE were found to be almost similar, i.e. 171.72 ± 4.13 µg QE/mg extract and 169.88 ± 9.79 µg QE/mg extract, respectively.

Determination of total flavonoid content

Antioxidant activity should not be concluded based on a single antioxidant test model. In practice, several *in vitro* test procedures are carried out for evaluating antioxidant activities with the samples of interest.[25] In the present study, we have evaluated antioxidant potential of CR HAE and CR CAE by various antioxidant assays including DPPH free radical scavenging assay, superoxide anion scavenging assay, ABTS radical scavenging assay, and oxygen radical absorbance capacity (ORAC) assay.

Antioxidant assays

1, 1-Diphenyl-2-picrylhydrazyl free radical scavenging assay

DPPH free radical scavenging assay is one of the most commonly used assays for testing preliminary radical scavenging activity of plant extracts. The antioxidant activity of plant extracts containing polyphenol components is due to their capacity to donate hydrogen atoms or electrons and to scavenge the free radicals.[26] Thus, the purple color of DPPH will be reduced to α, α- diphenyl-β-picrylhydrazine (yellow). Results indicate that CR HAE and CR CAE showed up to 85% of scavenging of DPPH free radicals [Figure 2]. Ascorbic acid was used as a positive control and showed scavenging activity up to 92%. CR HAE exhibited slightly higher antioxidant activity than CR CAE. EC_{50} values (µg/ml) were expressed in Table 2.

Phenazine methosulfate-NADH system superoxide anion scavenging assay

Although superoxide anion is a weak oxidant, it ultimately produces

Table 2: EC_{50} value of methanolic extracts of *Citrus reticulata* Blanco Peel for DPPH, superoxide, ABTS, anti-collagenase, and anti-elastase assays

Assay	EC_{50} value		
	Standard	CR HAE	CR CAE
DPPH (µg/ml)	4.07±0.19	250.33±40.16	254.73±15.78
Superoxide (µg/ml)	52.42±1.94	221.27±11.25	354.20±23.79
ABTS (µg/ml)	1.17±0.04	59.16±2.17	59.12±6.21
Anti-collagenase (µg/ml)	75.60±1.51	329.33±6.38	466.93±8.04
Anti-elastase (mg/ml)	0.01±0.001	3.22±0.24	5.09±0.30
ORAC value (µmoles TE*/g of substance)		1243	1063

*TE: TROLOX equivalent; DPPH: 1,1-Diphenyl-2-picrylhydrazyl; ORAC: Oxygen radical absorbance capacity; CR HAE: Hot Alcoholic Extract of *Citrus reticulata* Blanco; CR CAE: Cold Alcoholic Extract of *Citrus reticulata* Blanco

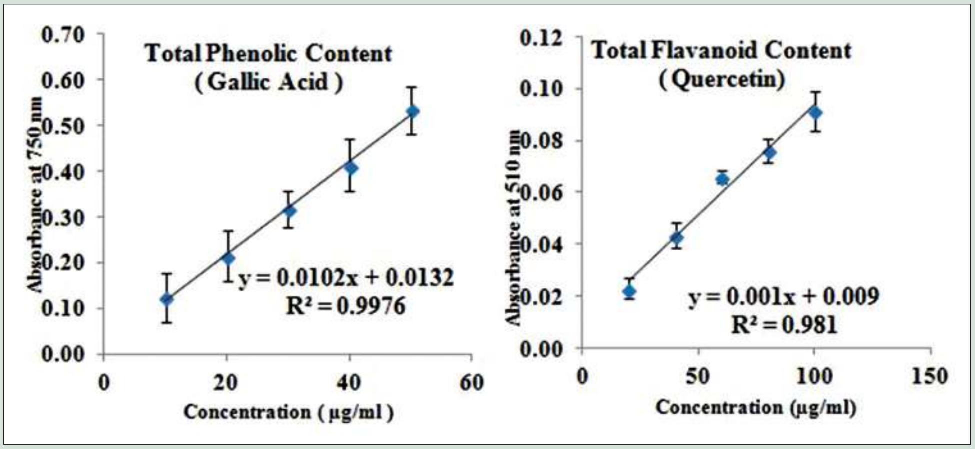

Figure 1: Standard curve for total phenolic and flavonoid content

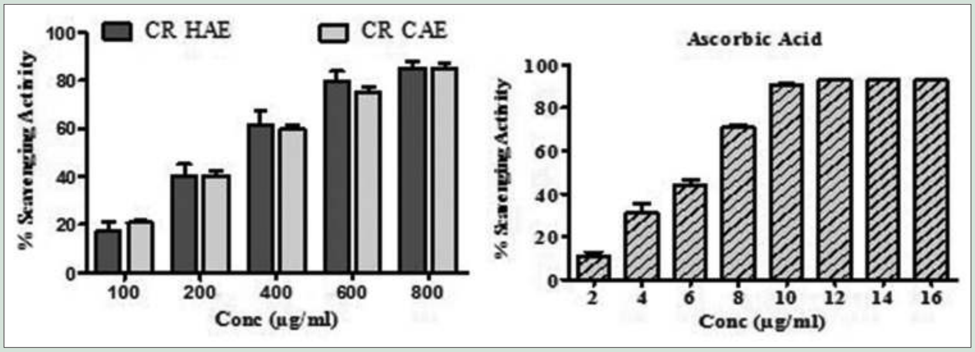

Figure 2: 1,1-Diphenyl-2-picrylhydrazyl free radical scavenging activity of ascorbic acid, CR HAE, and CR CAE

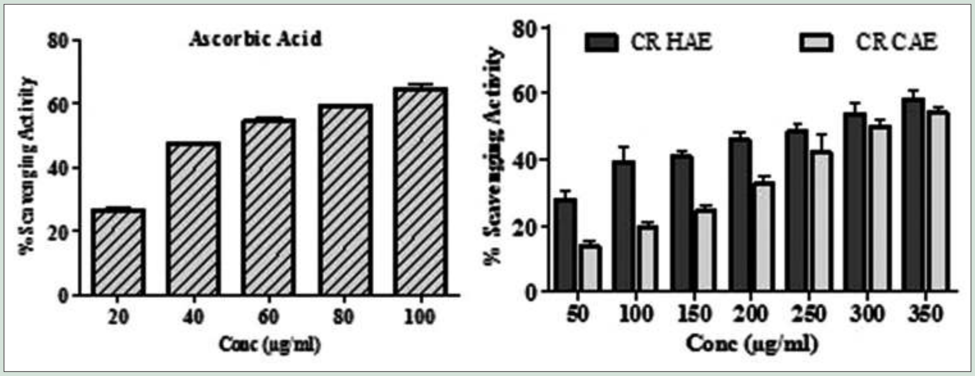

Figure 3: Superoxide anion radical scavenging activity of ascorbic acid, CR HAE and CR CAE

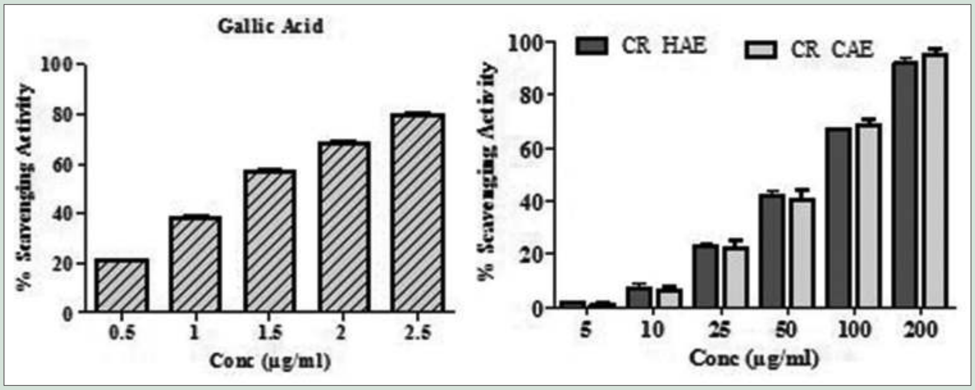

Figure 4: 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity of Gallic acid, CR HAE and CR CAE

powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress.[27] The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling and can be measured by their ability to reduce NBT. The decrease in absorbance at 560 nm with the plant extract indicates their ability to quench superoxide radicals in the reaction mixture. CR HAE showed strong superoxide scavenging activity than CR CAE [Figure 3]. Ascorbic acid was used as a positive control and scavenges superoxide anion up to 52%. EC_{50} values (µg/ml) were calculated and expressed in Table 2. CR HAE was found to be more effective ($P < 0.05$) than CR CAE as it showed less EC_{50} value (µg/ml) and potent antioxidant activity.

2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

ABTS assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in absorption, which can be followed spectrophotometrically.[28] ABTS activity was quantified in terms of percentage inhibition of the ABTS free radical cation by antioxidants in each sample. The ABTS values of the CR HAE, CR CAE, and gallic acid were presented in Table 2. CR HAE and CR CAE showed ABTS radical scavenging activity in concentration dependent manner and observed up to 59%. Gallic acid showed up to 79% of ABTS scavenging ability [Figure 4]. EC_{50} values (µg/ml) obtained for CR HAE and CR CAE indicated no significant difference ($P < 0.05$) and suggested that both the extracts have almost similar ABTS scavenging potential.

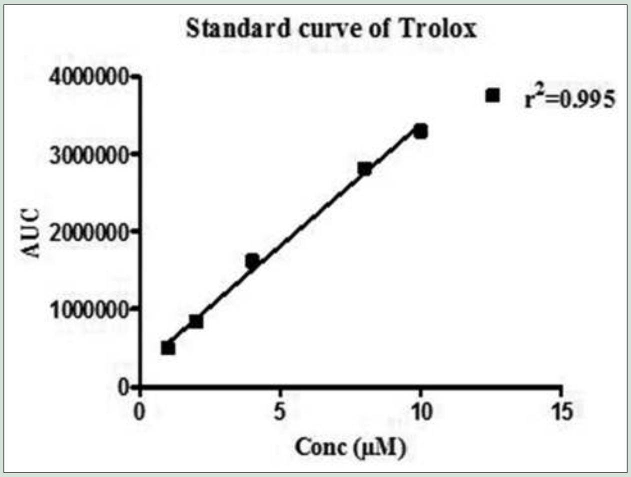

Figure 5: Standard curve of 6-hydroxy-2,5,7,8-tetra methylchromane-2-carboxylic acid for oxygen radical absorbance capacity assay

Oxygen radical absorbance capacity assay

ORAC assay is considered to be a more biologically relevant assay than other methods of measuring antioxidant potency because it measures the hydrogen-atom transfer reactions and simulates *in vivo* antioxidant action.[29] It also measures how well water-soluble and lipid-soluble components of a natural substance protect a standardized target from oxidation by peroxyl nitrite, hydroxyl radicals, superoxide anion, and

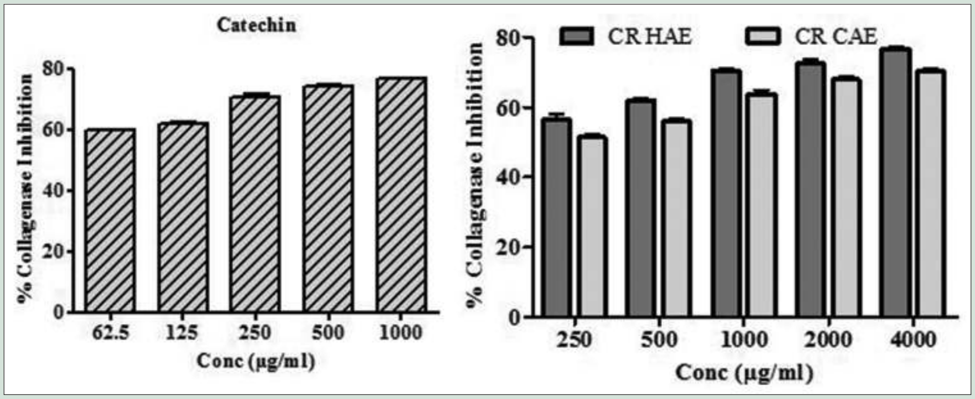

Figure 6: Collagenase inhibition activity of catechin, CR HAE and CR CAE

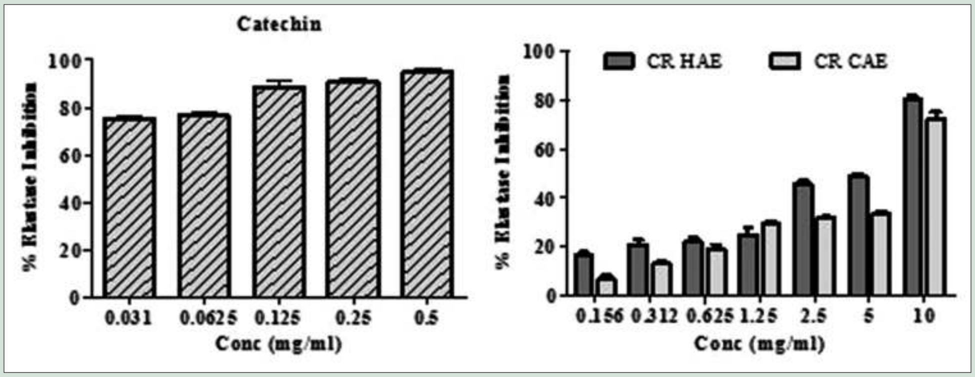

Figure 7: Elastase inhibition activity of catechin, CR HAE and CR CAE

singlet oxygen, and generates a score based on comparison with an antioxidant control.[30] In the present study, we have measured ORAC values for CR HAE and CR CAE and expressed in terms of TROLOX (mmoles TROLOX Equivalent/g of substance) [Figure 5]. CR HAE showed higher ORAC value (1243 mmoles TROLOX Equivalent/g of substance) than CR CAE (1063 µmoles TROLOX Equivalent/g of substance) [Table 2].

Enzyme assays

Anti-collagenase assay

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade various components of the ECM. Collagenase is one of the members of the MMP families and it is responsible for the degradation of collagen. Collagenase from the bacteria *C. histolyticum* (ChC) (C 3.4.24.3) is one of the few proteinases capable of degrading the triple-helical region of native collagen under physiological conditions and *in vitro* conditions using synthetic peptides as substrates. Protective effect of plant extracts against collagenase enzyme can be studied using ChC and synthetic substrate FALGPA.[31] In the present study, collagenase inhibition activity was evaluated for CR HAE and CR CAE. Catechin was used as a positive control. Results demonstrated that CR HAE extract was more effective in inhibiting collagenase enzyme than CR CAE and showed inhibition up to 76% [Figure 6]. Efficacy was measured in terms of EC_{50} value (µg/ml) and expressed in Table 2.

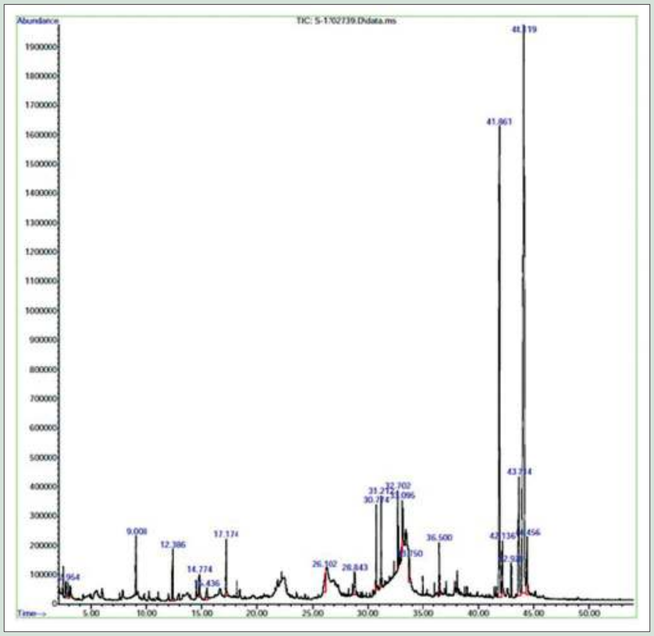

Figure 8: Gas chromatography-mass spectrometry chromatogram of hot alcoholic extract of *Citrus reticulata* Blanco

Anti-elastase assay

Elastase is the only enzyme capable of degrading elastin. Inhibition of elastase enzyme can retain the elasticity and suppleness of skin.[32] In terms of anti-aging, finding inhibitors of elastase enzymes can be useful to prevent loss of skin elasticity and thus skin sagging. In anti-elastase assay, porcine pancreatic elastase was assayed spectrophotometrically by

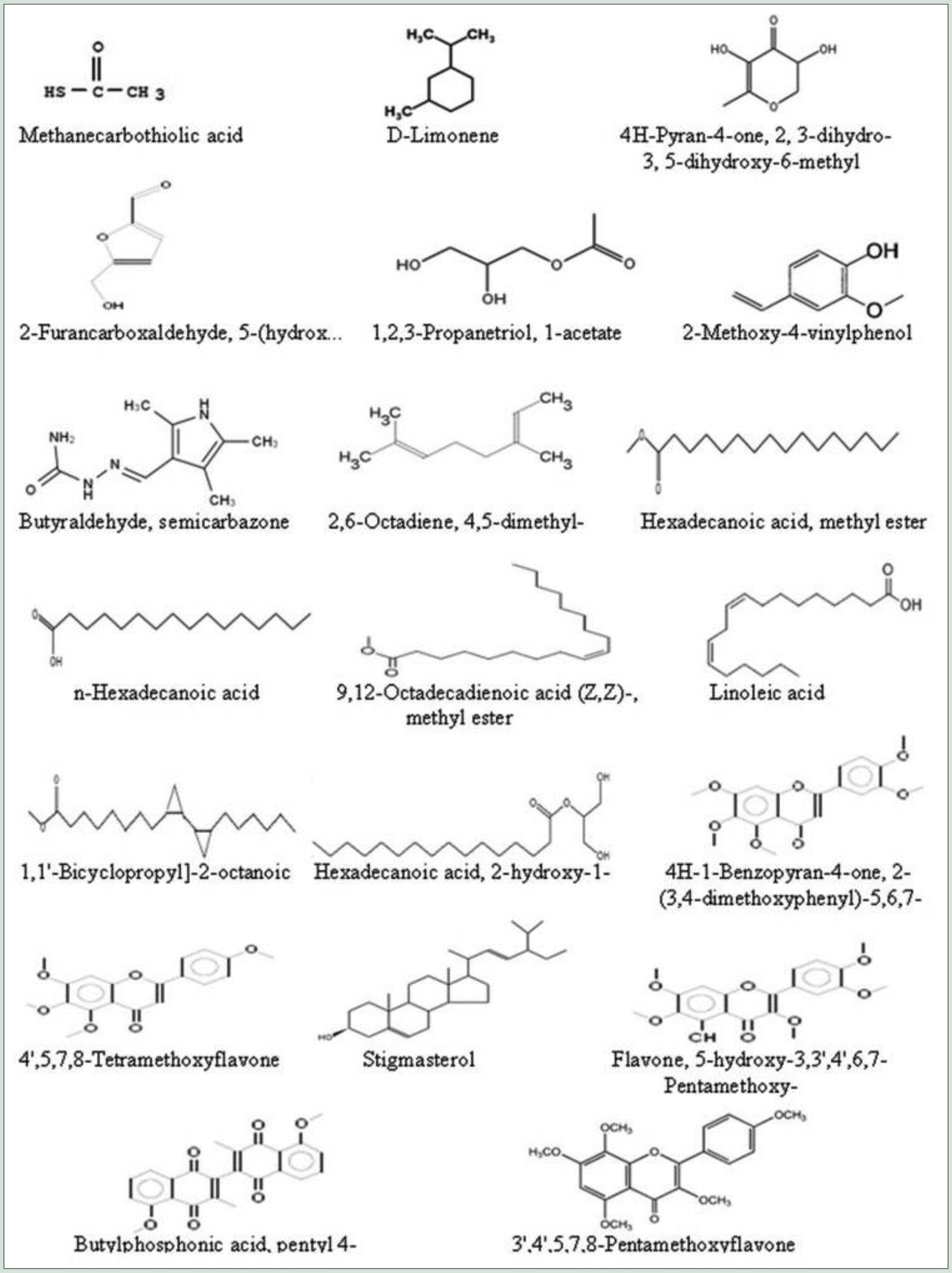

Figure 9: Structures of compounds identified in gas chromatography-mass spectrometry analysis of hot alcoholic extract (CR HAE) of *Citrus reticulata* Blanco

using [*N*-Succ-(Ala) 3-*p*-nitroanilide] as the substrate, and the amount of *p*-nitroaniline was determined by measuring the absorbance at 410 nm. The inhibitory effects of CR HAE and CR CAE on elastase activity were investigated. Catechin was used as a positive control. CR HAE showed strong % elastase inhibition activity compared to CR CAE and up to 80% inhibition was observed [Figure 7]. EC_{50} values (mg/ml) were expressed in Table 2 and it was observed that CR HAE was more potent than CR CAE ($P < 0.05$). EC_{50} value obtained for catechin was found to be very less indicating its strong anti-elastase activity and its role as a strong anti-aging component. Kim *et al*., 2004 showed that the catechin and epigallocatechin gallate isolated from green tea (*Camellia sinensis*) were potent anti-elastase inhibitors.

Gas chromatography-mass spectrometry analysis

CR HAE showed higher antioxidant and anti-aging abilities compared to CR CAE; hence, GC-MS analysis of CR HAE was carried out to understand the constituents present in it. A total of 20 different compounds were identified and presented in Table 3. The chromatogram showed 20 peaks in the retention time ranged from 2.95 min to 44.45 min [Figure 8]. The largest peak at 44.11 min with 44.37% area was identified as butylphosphonic acid, pentyl 4-(2-phenylprop-2-yl) phenyl ester. Zab *et al*., 2012[33] reported the antioxidant and anti-tumor activity of butylphosphonic acid, pentyl 4-(2-phenylprop-2-yl) phenyl ester present in the ethanolic extract of *C. reticulata*. Another major compound found was 4H-1-Benzopyran-4-one, 2-(3, 4-dihydro) or 4-Chromanone (21.43% area) which is a type of

Table 3: GC-MS analysis of Hot Alcoholic Extract (CR HAE) of *Citrus reticulata* Blanco

Retention time	Area (%)	Compound name	Molecular weight	Molecular formula
2.95	0.73	Methanecarbothiolic acid	76	C_2H_4OS
9.00	1.46	D-limonene	136	$C_{10}H_{16}$
12.38	2.12	4H-pyran-4-one, 2, 3-dihydro-3, 5- dihydroxy-6-methyl	144	$C_6H_8O_4$
14.77	1.08	2-furancarboxaldehyde, 5-(hydrox…)	126	$C_6H_6O_3$
15.43	0.98	1,2,3-propanetriol, 1-acetate	134	$C_5H_{10}O_4$
17.17	1.67	2-Methoxy-4-vinylphenol	150	$C_9H_{10}O_2$
26.10	2.46	Butyraldehyde, semicarbazone	72	C_4H_8O
28.84	1.89	2,6-octadiene, 4,5-dimethyl-	138	$C_{10}H_{18}$
30.77	1.54	Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$
31.21	2.51	n-hexadecanoic acid	256	$C_{16}H_{32}O_2$
32.70	1.32	9,12-octadecadienoic acid (Z, Z)-, methyl ester	294	$C_{19}H_{34}O_2$
33.09	1.12	Linoleic acid	280	$C_{18}H_{32}O_2$
33.75	0.98	[1,1'-bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	322	$C_{21}H_{38}O_2$
36.50	1.05	Hexadecanoic acid, 2-hydroxy-1-	286	$C_{17}H_{34}O_3$
41.86	21.43	4H-1-benzopyran-4-one,2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxy-	372	$C_{20}H_{20}O_7$
42.13	2.12	4',5,7,8-tetramethoxyflavone	342	$C_{19}H_{18}O_6$
42.92	1.18	Stigmasterol	278	$C_{18}H_{30}O_2$
43.71	1.25	Flavone, 5-hydroxy-3,3',4',6,7-pentamethoxy-	388	$C_{20}H_{20}O_8$
44.11	44.37	Butylphosphonic acid, pentyl 4-	402	$C_{24}H_{35}O_3P$
44.45	2.84	3',4',5,7,8-pentamethoxyflavone	372	$C_{20}H_{20}O_7$

GC-MS: Gas chromatography-mass spectrometry; CR HAE: Hot Alcoholic Extract of *Citrus reticulata*; CR CAE: Cold Alcoholic Extract of *Citrus reticulata*

flavanone. Di Majo *et al*., 2005[34] isolated the flavanones from citrus fruits and showed structure-dependent antioxidant activity. Other compounds 3', 4', 5, 7, 8-Pentamethoxyflavone and 4', 5, 7, 8-Tetramethoxyflavone are types of polymethoxyflavone (PMF). PMF exhibit a broad spectrum of biological activities. Recently, Ho *et al*., 2012[35] isolated and identified hydroxylated PMFs from citrus peels and investigated their biological activities, including anti-inflammation and cancer chemopreventive property. Other compounds identified include D-Limonene (1.46% area), 4H-Pyran-4-one, 2, 3-dihydro-3, 5- dihydroxy-6-methyl (1.46% area), 2-Methoxy-4-vinylphenol (1.67% area), and n-Hexadecanoic acid (2.51% area) and could be related with the anti-aging potential of *C. reticulata* Blanco peel. Structures of all identified compounds were presented in Figure 9.

CONCLUSION

C. reticulata Blanco extracts were evaluated against skin aging through *in vitro* antioxidant, anti-collagenase, and anti-elastase assays. The study showed that methanolic extract (Soxhlation) exhibited promising antioxidant and anti-enzymatic activity and can be used as a potent anti-wrinkle agent in anti-aging skin care formulations.

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Conflicts of interest

There are no conflicts of interest.

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