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Research Article

IN VITRO SCREENING OF ANTIOXIDANT AND ANTIAGING POTENTIAL OF *CUCUMIS SATIVUS* FRUIT EXTRACT

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ABSTRACT

Objective: The present study was designed to screen the anti-aging and anti-wrinkle potential of *Cucumis sativus* fruit through *in vitro* estimation of antioxidant, anti-hyaluronidase, anti-elastase, anti-collagenase/anti-matrix metalloproteinase (MMP)-1, and anti-tyrosinase activity.

Methods: Raw juice of cucumber was taken, filtered and fractionated with ethyl acetate and n-butanol. The obtained extracts were then evaluated for their antioxidant potential through 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay taking ascorbic acid as positive control and other enzymatic activities in reference to hyaluronidase inhibition, MMP-1/collagenase inhibition, and elastase inhibition taking catechin as reference standard whereas for tyrosinase inhibition the standard used was quercetin.

Results: All the evaluations were performed in triplicates and results were noted down. It was observed that aqueous extract of *C. sativus* fruits showed a maximum DPPH radical scavenging activity ($p<0.0001$), half-maximal inhibitory concentration (IC_{50}) at a concentration of 122.67 µg/ml. The ethyl acetate fraction of *C. sativus* fruits exhibited maximum hyaluronidase ($p<0.0001$), MMP-1/collagenase ($p<0.04$), and tyrosinase ($p<0.04$) inhibitory activity, IC_{50} at a concentration of 59.54, 45.79, and 24.46 µg/ml, respectively. The elastase ($p<0.0001$) inhibitory activity by n-butanol fraction of *C. sativus* fruits extract was maximum, IC_{50} at a concentration of 52.76 µg/ml.

Conclusion: A potent anti-aging and anti-wrinkle properties were well demonstrated by *C. sativus,* as depicted from the results obtained.

Keywords: Anti-aging, Anti-wrinkle, *Cucumis sativus*, Antioxidant, Hyaluronidase, Elastase, Matrix metalloproteinase-1, Tyrosinase.

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INTRODUCTION

Aging is a progressive process influenced by both intrinsic and chronological factors and extrinsic or environmental factors which include ultraviolet (UV) rays, air pollution, stress, or smoking [1]. Excessive exposure to UV rays results in the formation of reactive oxygen species (ROS) which causes the adverse effects on the dermal and epidermal connective tissues leading to the damage to cell and cell membranes [2]. Skin physiological changes occurring due to the acceleration of the free radicals, cell contents, and lipid peroxidation stimulated by high exposure to UV radiation directly cause skin aging [3]. Skin shows rugged appearance, hyperpigmentation, dark spots, deep wrinkles, etc. [4]. Hyaluronic acid, elastin, and collagen are important for maintaining skin structure, moisture content, elasticity, and strength of connective tissue of the skin which is decreased in the aging skin [5-7]. The increase in the activity of hyaluronidase, elastase, and collagenase/matrix metalloproteinase (MMP)-1 is responsible for decrease in the strength of connective tissue, water holding capacity of the skin, and elasticity of skin. Excessive exposure to UV radiations also results in the development of dark spots which may be due to the overproduction of melanin [8]. Tyrosinase is the main enzyme that catalysis melanin synthesis in melanocytes [9]. Inhibition of these enzymes plays an important role in skin aging. Since traditional medicines and plants provide an extensively large unexplored source for the development of new, potent, and safe cosmetic and skincare products, researchers have paid attention on the exploration of plants and plant extracts for combating skin aging [2]. In the present study, *Cucumis sativus* commonly known as cucumber was studied for its anti-aging and anti-wrinkle potential. It is a commonly used vegetable crop which belongs to the Cucurbitaceae family [10]. Conventionally, it has been used widely for various skin problems and applied topically for swollen eyes, burns, dermatitis, antioxidant, skin whitening and anti-wrinkle effect, analgesic, anticancer, and hypoglycemic activity [11,12]. Chemical composition of cucumber fruit includes α-linolenic acid, Caffeic acid, Citrulline, Vitamin C, Cucurbitin B, β amyrin, β sitosterol, Chlorogenic acid, Cucurbitin A, Cucurbitin C, Cucurbitin E, Ferulic acid, Folacin, Hexanal, Pentadec-cis-8-en-1-al, Mevalonic acid, Rubidium, Strontium, γ-glutamyl-beta-pyrazole-1-yl-alanine, Hexen-(2)-al-(1), Propanal, Myristic acid, Squalene, and Zirconium [13]. Taking into accounts the above aspects, the present study was designed to evaluate the anti-aging and anti-wrinkle potential of *C. sativus* fruit juice.

METHODS

Collection and authentication of plant materials

The fruits of *C. sativus* were collected from the local market of Rohtak in the month of October-November 2017 and authenticated from the Department of Botany Maharshi Dayanand University, Rohtak. The voucher specimen (VS/Ph cog/2017/401) of *C. sativus* is kept in the Department of Pharmaceutical Sciences, Maharshi Dayanand University, Rohtak.

Chemicals

1, 1-diphenyl-2-picryl-hydrazyl (DPPH), Tris-HCL buffer, Tricine buffer, Catechin, Tris-HCL buffer, Bovine Hyaluronidase enzyme, Collagenase from *Clostridium histolyticum,* porcine pancreatic elastase, Furylacryloyl-Leucine-Glycine-Propyl-Alanine (FALGPA), N-(methoxysuccinyl)–ala-ala-pro-val 4-nitroanilide (MAAPVN), Albumin, Tyrosinase, Sodium hyaluronate, acetate buffer, dimethyl sulfoxide (DMSO), calcium chloride, and p-dimethyl amino benzaldehyde (PDMAB) were procured from Sigma Aldrich and Fluka chemicals. Other chemicals and reagents were of analytical grade which was used in the present evaluation.

Extraction and fractionation of plant material

Two kilograms fruits of *C. sativus* were taken and washed properly. Then peeled off and seeds were removed and juice was collected

in juicer. The juice of fruits was fractionated with ethyl acetate and n-butanol (150 ml each) and separated the resultant portions using separating funnel and aqueous portion left in the funnel. The obtained fractions were evaporated and concentrated to get the respective extracts. The extractive values of all the fractions are depicted in Table 1.

Antioxidant studies

DPPH radical scavenging activity

The antioxidant potential of the extract was based on the inhibitory potential of the stable DPPH free radical and the procedure followed was as explained by Braca *et al*. [14] with little changes. The extracts of *C. sativus* were prepared in a dilution series (100, 200, and 300 µg/ml) in DMSO. The reaction mixture consisted of 0.1mL test sample with 0.2mL DPPH solution (0.15 mM in 80% methanol solution). The final reaction mixture was shaken dynamically and kept at room temperature for 30 min in the dark. Ascorbic acid was used as a standard. The evaluations were done spectrophotometrically (using ELISA reader) at 517 nm and the % scavenging potential was calculated by the given formula:

Inhibitory activity (%) = (Abs control–Abs sample\Abs control)×100.

Where Abs is the absorbance

The antioxidant potential of the extracts was demonstrated as half-maximal inhibitory concentration (IC_{50}) (a concentration [µg/ml] which 50% reduction in the formation of DPPH free radicals are known as IC_{50} value of any drug or plant extract). All the observations were taken in the triplicates, graph was plotted by average.

Enzymatic studies

Hyaluronidase inhibitory activity

Fifty microliter bovine hyaluronidase (prepared by diffusing 7900 units/ml of enzyme in 0.1 M acetate buffer; pH 3.5) was added to 50 µl of different dilutions of test extract prepared using 5% DMSO and incubated at 37°C for 20 min. In the control group, 50 µl of DMSO was added instead of plant extract. Hyaluronidase was activated by the addition of 50µl of calcium chloride (12.5 mM) in reaction mixture and the whole mixture was incubated for 20 min at 37°C. The obtained Ca+2 activated hyaluronidase was then treated with 250 µl of sodium hyaluronate (0.1 M acetate buffer; pH 3.5, was used to diffuse 1.2 mg/ml) and kept for 3 min on a water bath at 100°C. The reaction mixture was then cooled to room temperature and added 1.5 ml of PDMAB ([4 g] dissolved in glacial acetic acid [350 ml] and 10 N HCl [50 ml]). The reaction mixture was then kept for 20 min at room temperature [15]. The absorbance was measured at 585nm. Inhibitory effect was calculated as:

Inhibition (%) = ([Abs of control–Abs of sample]/Abs of control)×100

Abs = Absorbance

MMP-1/Collagenase inhibitory activity

MMP-1/collagenase inhibition assay was carried out on the basis of the method discussed by Kim *et al*. [16] It involves the use of collagenase (0.8 units/ml) from *C. histolyticum* and 2 mM of synthetic substrate, FALGPA. The final reaction mixture contained 25 µl of 50 mM tricine buffer, 25 µl of the test sample, and 25 µl of collagenase enzyme (0.1 units). After the addition of 50 µl of 2 mM FALGPA, absorbance was immediately measured at 340 nm by ELISA reader. Catechin was used as a standard. The % enzyme inhibition was calculated by the given formula:

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

Where A= activity of control, B=activity of test extracts.

Elastase inhibitory activity

Elastase inhibition activity was analyzed by the procedure discussed by Pientaweeratch *et al*. [17] Mix 0.1 ml of a 0.2 M Tris-HCl buffer having 1% albumin, 0.025 ml of 10 mM MAAPVN and 0.05 ml of the sample of different concentrations (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml). Then add 0.025 ml of elastase (3 units/ml) to each test sample. Incubate the resultant reaction mixture at 25°C for 20 min and measure the absorbance by ELISA reader at 410 nm. Percentage inhibition was calculated using following formula:

Inhibition rate (%) = (l-[C-D]/[A-B]) ×100
Where,
A and B represent the absorbance without a test sample after and before incubation, respectively,
C and D represent the absorbance with a test sample after and before incubation, respectively.

Tyrosinase inhibitory activity

The determination of tyrosinase was performed using L-DOPA as substrate [18-21]. First, dissolve 0.8 ml of L-DOPA (2.5 mM) with 2.4 ml of phosphate buffer solution (0.067 M) of pH 6.6. Then incubate the reaction mixture for 10 min at 37sC. After this, 0.8 ml of 2 mg/ml extracts and 0.8 ml tyrosinase solution were mixed. The solution was instantaneously examined for the generation of dopachrome by estimating the linear rise in optical density for 5 min at 475 nm. The observations were taken in triplicates. The tyrosinase inhibitory activity was estimated by the formula:

$$\text{Inhibition}(\%)=\frac{(C-D)(A-D)}{C-D}$$

Where, A and B are the absorbance of a sample with L-DOPA and without L-DOPA, respectively,
C is the absorbance without sample and with substrate, and D is the absorbance without both sample and substrate.

Statistical analysis

Data are expressed as IC_{50}. Linear regression was used to determine the IC_{50} values.

RESULTS

Determination of extractive value

In the present study, the fruits of *C. sativus* were collected and juice was prepared followed by fractionation using ethyl acetate and n-butanol. The extracts were dried; stored and extractive value was evaluated. The results are summarized in Table 1.

***In vitro* DPPH radical scavenging activity**

The absorbance of all extracts was measured at 517 nm for different concentrations. The percentage antioxidant activity of ethyl acetate, N-butanol, and aqueous extract of *C. sativus* at 300 µg/ml exhibited the maximum antioxidant potential of 47.13, 49.64, and 72.40 µg/ml, respectively, while ascorbic acid at 300 µg/ml concentration exhibited the antioxidant potential of 91.11. *C. sativus* extracts (ethyl acetate, N-butanol, and aqueous) have dose-dependent significant ($p<0.0001$) DPPH radical inhibitory activity with IC_{50} value, at a concentration of 358.04, 294.64, and 122.67 µg/ml, respectively, in comparison to ascorbic acid (IC_{50}) at 24.57µg/ml. Fig. 1 shows the DPPH radical inhibitory potential of *C. sativus* extracts as compared to ascorbic acid.

Table 1: % yield obtained for different extracts of *Cucumis sativus*

S. No	Extracts	Extractive value (% W/W)
1.	Ethyl acetate	0.035
2.	n-butanol	0.085
3.	Water	1.35

Hyaluronidase inhibitory activity

The absorbance of all extracts was read at 585 nm for different concentrations and the percentage of the total anti-hyaluronidase potential of ethyl acetate, n-butanol, and aqueous extracts of *C. sativus* was calculated. The ethyl acetate, n-butanol, and aqueous extracts of *C. sativus* at 100 µg/ml exhibited a maximum total hyaluronidase inhibition (Fig. 2) of 58.73, 88.59, and 59.15. The IC_{50} value of the ethyl acetate (59.54 µg/ml), n-butanol (377.28 µg/ml), and aqueous extracts (75.84 µg/ml) of *C. sativus* was found to be significant ($p<0.0001$), as compared to standard catechin having the IC_{50} value of 14.57µg/ml.

Elastase inhibitory activity

The absorbance of all extracts was read at 410 nm for different concentrations and the percentage of total anti-elastase potential of ethyl acetate, n-butanol, and aqueous extracts of *C. sativus* was calculated. The ethyl acetate, n-butanol, and aqueous extracts of *C. sativus* at 100 µg/ml exhibited a maximum total elastase inhibition (Fig. 3) of 71.54, 76.98, and 67.80 µg/ml, respectively. The IC_{50} value of the ethyl acetate (52.76 µg/ml), n-butanol (42.16 µg/ml), and aqueous (66.08 µg/ml) extracts of *C. sativus* was found to be significant ($p<0.03$) in comparison to standard catechin having the IC_{50} value 1.84 µg/ml.

MMP-1/Collagenase inhibitory activity

The absorbance of all extracts was read at 340 nm for different concentrations and the percentage of the total anti-collagenase potential of ethyl acetate, n-butanol, and aqueous extracts of *C. sativus* was calculated. The ethyl acetate, n-butanol, and aqueous extracts of *C. sativus* at 100 µg/ml exhibited a maximum total Collagenase inhibition (Fig. 4) of 69.06, 67.12, and 65.09 µg/ml, respectively. The IC_{50} value of the ethyl acetate (45.7 9 µg/ml), n-butanol (50.59 µg/ml), and aqueous (56.88 µg/ml) extracts of *C. sativus* was found to be significant ($p<0.04$) in comparison to standard catechin having the IC_{50} value 18.21 µg/ml.

Tyrosinase inhibitory activity

The absorbance of all extracts was read at 492 nm for different concentrations and the percentage of the total anti-tyrosinase potential of ethyl acetate, n-butanol, and aqueous extracts of *C. sativus* was calculated. The ethyl acetate, n-butanol, and aqueous extracts of *C. sativus* at 100 µg/ml exhibited a maximum total tyrosinase inhibition (Fig. 5) of 77.21, 87.03, and 79.01 µg/ml. The IC_{50} value of the ethyl acetate (24.46 µg/ml), n-butanol 99.68 µg/ml), and aqueous (40.09 µg/ml) extracts of *C. sativus* was found to be significant ($p<0.04$) as compared to standard quercetin having the IC_{50} value 18.27 µg/ml.

IC_{50} of *C. sativus* extracts for antioxidant assay and all enzymatic assays are shown in Table 2.

DISCUSSION

Photoaging is mainly stimulated by the excessive exposure of skin to UV radiations, which causes the release of ROS, thereby rendering

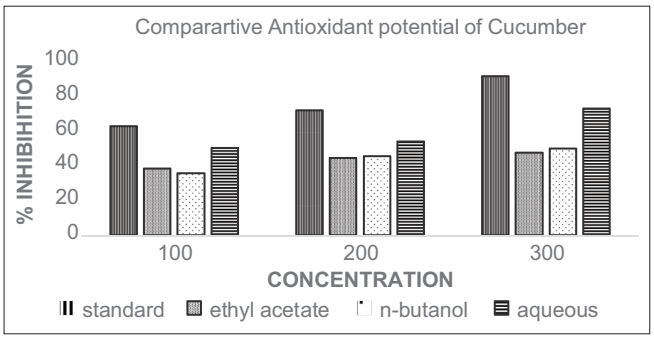

Fig. 1: 1, 1-diphenyl-2-picryl-hydrazyl radical inhibitory potential of *Cucumis sativus* extracts to compare with ascorbic acid. All values were found to be significant (p<0.0001)

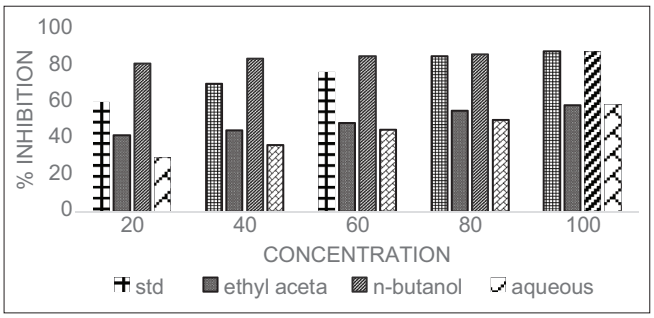

Fig. 2: Hyaluronidase inhibitory potential of *Cucumis sativus* extracts to compare with catechin. All values were found to be significant (p<0.0001)

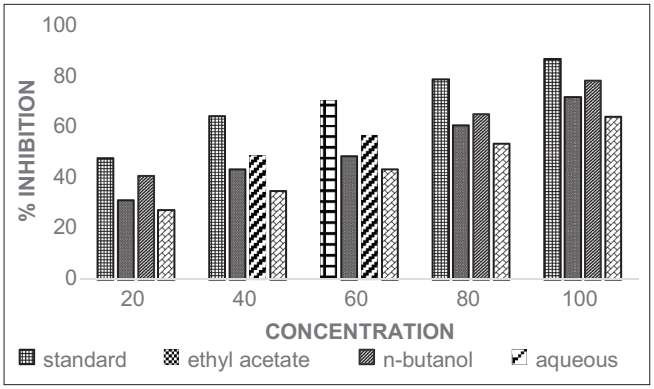

Fig. 3: Elastase inhibitory potential of *Cucumis sativus* extracts to compare with catechin. All values were found to be significant (p<0.03)

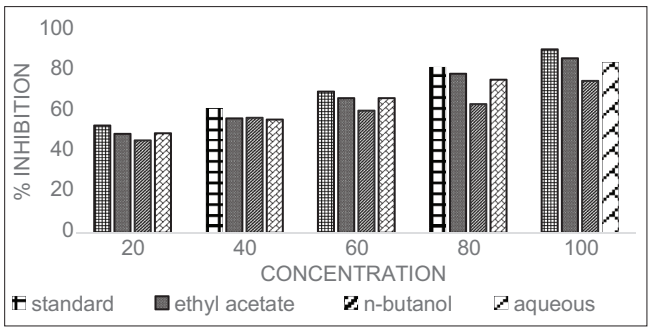

Fig. 4: Collagenase inhibitory potential of *Cucumis sativus* extracts to compare with catechin. All values were found to be significant (p<0.04)

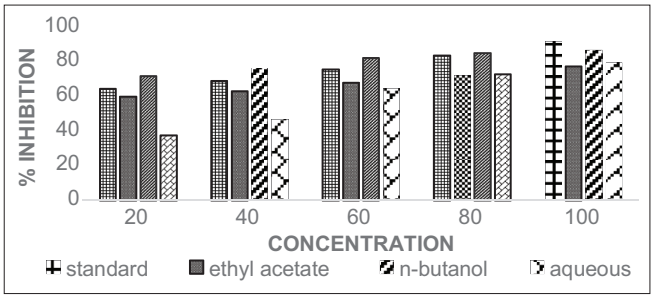

Fig. 5: Tyrosinase inhibitory potential of *Cucumis sativus* extracts to compare with quercetin. All values were found to be significant (p<0.04)

Table 2: IC_{50} of extracts of *Cucumis sativus* fruit in antioxidant and other enzymatic assays

S. No	Assay	IC_{50} (µg/ml)			
		Standard	Ethyl acetate extract	N-butanol extract	Aqueous extract
1.	Antioxidant assay	24.57	358.04	294.64	122.67
2.	Hyaluronidase inhibition assay	14.57	59.54	377.28	75.84
3.	Elastase inhibition assay	1.84	52.76	42.16	66.08
4.	MMP-1/collagenase inhibition assay	18.21	45.79	50.59	56.88
5.	Tyrosinase inhibition assay	18.27	24.46	99.68	40.09

IC_{50}: Half-maximal inhibitory concentration

severe damage to proteins, lipids, and DNA. *C. sativus* flesh mainly consists of water, ascorbic acid (Vitamin C), gallic acid, caffeic acid, flavonoids, phenolic compounds, etc., and is known to be used in many skincare products to reduce the signs of skin aging and relieve skin irritations [22]. In the present study, the anti-aging and anti-wrinkle potential of *C. sativus* fruit was studied through *in vitro* estimation of antioxidant, anti-hyaluronidase, anti-elastase, anti-collagenase/anti-MMP, and anti-tyrosinase activity. The aqueous extract of *C. sativus* was fractionated into ethyl acetate and n-butanol fraction to correlate the possible constituents responsible for the observed activities. The maximum antioxidant activity was exhibited by the aqueous fraction as it is rich in ascorbic acid, cucurbitacin a, b, c, and 96% water. The ethyl acetate fraction contains organic compound such as citric acid, malic acid, gallic acid, caffeic acid, and α-linoleic acid and may be responsible for the anti-hyaluronidase, anti-collagenase/anti-MMP, and anti-tyrosinase activity. The elastase inhibition was maximum shown by the n-butanol fraction. This may be attributed to the presence of phenolic compound such as p-hydroxybenzoic acid, hydroxycinnamic acid, flavones, β- carotene, flavanols, squalene, and β- sitosterol. The presence of carotenoids, phenolic flavonoids, tannins, polyphenols, and lycopene was confirmed by the various phytochemical screening studies carried out by different researchers [23-25]. The main acids found are citric and malic acids. Phenolic compounds present are p-hydroxybenzoic acid, hydroxycinnamic acid, flavones, and flavonols. Cucumber is rich in Vitamin C and Vitamin B [26].

CONCLUSION

From the above results, it was concluded that the lyophilized extracts of *C. sativus* are potent inhibitors of the hyaluronidase, elastase, MMP-1/collagenase, and tyrosinase enzymes. It also has potential to inhibit the free radicals. Therefore, it can be accomplished that *C. sativus* plays important role in the skincare products and can be investigated further for its use in cosmetics industries.

AUTHORS' CONTRIBUTIONS

1. Dr. Chanchal Garg: Designed the project and performed the analytical part of the research
2. Mr. Ravinder: Prepared the plant extract
3. Prof. Munish Garg: Provide supervision and guidance in this research work.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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