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Phytochemical and Nutritional Characterization of *Withania coagulans* Fruit Extract with Antioxidant, Anti-Glycation and Calcium Oxalate Crystallization-inhibitory Activities

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Withania coagulans has been traditionally used in ethnomedicine for the management of metabolic and urinary disorders; however, studies correlating its nutritional composition with antioxidant, anti-glycation and calcium oxalate crystallization activities remain limited. This study evaluated the phytochemical profile, nutritional composition and multifunctional bioactivities of ethanolic extract of *W. coagulans* fruit using *in vitro* models. Preliminary phytochemical screening, proximate and micronutrient analysis and GC-MS characterization were performed. Antioxidant activity was assessed using DPPH radical scavenging and potassium ferricyanide reducing-power assays, while anti-glycation and CaOx crystallization-inhibitory activities were evaluated using the BSA-fructose glycation model and calcium oxalate crystallization assays, respectively. Phytochemical analysis revealed the presence of flavonoids, alkaloids, phenolics, glycosides and phytosterols. The extract showed appreciable total phenolic (75.6 mg catechol equivalents/g extract) and flavonoid contents (60 mg quercetin equivalents/g extract). Nutritional analysis demonstrated the presence of carbohydrates, dietary fibre, vitamin E, potassium and calcium. GC-MS profiling identified fatty acid esters and lipid-derived phytoconstituents as major components. The extract exhibited concentration-dependent antioxidant activity with IC_{50} value of 170.8 µg/mL in DPPH assay. It also demonstrated moderate anti-glycation activity and effectively inhibited calcium oxalate nucleation and aggregation *in vitro*.

Keywords: *Withania coagulans*, Antioxidant activity, Anti-glycation, Calcium oxalate, Crystallization-inhibitory activity.

INTRODUCTION

In recent years, plant-derived bioactive compounds have received growing attention for their possible role in managing disorders associated with oxidative stress. Phenolic compounds, flavonoids, alkaloids and phytosterols have been investigated for their antioxidant and anti-glycation activities, as well as their effects on calcium oxalate (CaOx) crystallization [1-3]. Medicinal plants also contain nutrients and other phytochemicals that may be useful in developing functional food ingredients. Their value lies not only in the presence of individual compounds but also in the range of biological activities that their extracts may exhibit.

Oxidative stress develops when reactive oxygen species (ROS) accumulate beyond the capacity of the body's antioxidant defense system, which cause damage to lipids, proteins and nucleic acids [4,5]. This imbalance has been linked to several disorders, for example, diabetes mellitus, nephropathy, cardiovascular diseases and urolithiasis. Its connection with both metabolic disturbances and kidney stone formation makes the investigation of natural antioxidants relevant to these conditions.

The formation of advanced glycation end products (AGEs) is another process associated with metabolic complications and oxidative damage. AGEs arise from non-enzymatic reactions between reducing sugars and biological molecules, particularly proteins and lipids. Their accumulation can alter the structure and function of these molecules and contribute to tissue damage. Binding of AGEs to their receptor (RAGE) activates cellular pathways associated with inflammation and oxidative stress, which may aggravate renal injury and diabetes related complications [6,7]. Plant extracts with antioxidant and anti-glycation properties may help limit these processes and warrant further investigation as potential sources of bioactive compounds.

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Oxidative stress has been linked to calcium oxalate (CaOx) stone formation, where crystal nucleation, growth, aggregation and retention in the urinary tract determine the progression of urolithiasis. ROS can damage renal epithelial cells and favour crystal adhesion and retention, which develop conditions that may support stone development [8,9]. The examining the effects of plant extracts on CaOx nucleation and aggregation may help clarify their role in regulating crystal formation and clumping. Evaluating these activities alongside antioxidant and anti-glycation properties provides a basis for examining their potential relevance to renal health, although *in vitro* findings alone cannot establish efficacy in preventing kidney stones.

Withania coagulans (Stocks) Dunal, a member of the Solanaceae family, is traditionally used to manage diabetes, inflammation, urinary disorders and liver diseases. Its reported constituents, which include withanolides, flavonoids, alkaloids, phenolics and steroidal lactones [10]. Although its phytochemical and antioxidant properties have been studied, the effects of its fruit extract on calcium oxalate (CaOx) nucleation and aggregation remain insufficiently explored. The present study investigates the ethanolic fruit extract of *W. coagulans* for its phytochemical and nutritional composition, antioxidant and anti-glycation activities and effects on CaOx crystallization through *in vitro* assays. The CaOx nucleation and aggregation were assessed separately and crystal morphology was examined microscopically. GC-MS analysis was used to the detectable volatile and semi-volatile constituents of the extract. These analyses were used to explore the relationship between the chemical composition of extract and its antioxidant, anti-glycation and CaOx crystallization related activities.

EXPERIMENTAL

Dried fruits of *Withania coagulans* were procured from a local Ayurvedic herbal supplier in Kumbakonam, India. The plant was authenticated and assigned the authentication number CARISM00316 by the Centre of Advanced Research in Indian System of Medicine, SASTRA Deemed University, Thanjavur. The collected fruits were cleaned to remove extraneous materials, shade-dried if necessary and stored in airtight containers under dry conditions until further use.

Preparation of plant extract: The dried fruits of *W. coagulans* were mechanically powdered and 50 g of coarse powder was subjected to successive cold maceration using chloroform, ethyl acetate and ethanol in increasing order of solvent polarity. Extraction was performed at room temperature for 72 h using a solvent-to-sample ratio of 10:1 (v/w) with intermittent shaking. The extracts were filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator at 40-45 ºC to obtain semi-solid crude extracts. The concentrated extracts were stored at 4 ºC until further analysis. Percentage yield was calculated based on the initial dry weight of the powdered material. Among the extracts, the ethanolic extract exhibited comparatively higher extractive yield, broader phytochemical distribution and superior preliminary biological activity and was therefore selected for subsequent investigations [11,12]. From 50 g of powdered plant material, the ethanolic extract of *W. coagulans* fruits produced 1.2 g (yield: 2.4% (w/w)) of concentrated extract. To reduce the deterioration and preserve sample stability, the concentrated extract was moved into sealed containers and kept at 4 ºC until additional analysis. The ethanolic extract was employed for phytochemical characterization, antioxidant, anti-glycation, calcium oxalate crystallization tests and GC-MS analysis. The dried fruit powder was used for the separate proximate and mineral composition analyzes, which are therefore expressed on a dried fruit material basis.

Qualitative phytochemical analysis: The initial qualitative phytochemical investigation was performed using routine procedures to determine the existence of several bioactive compounds, as described elsewhere [13].

Gas chromatography-mass spectroscopy analysis: GC-MS analysis was performed using a Shimadzu QP-2010 Plus system equipped with an AOC-20i autosampler and mass spectrometer. A sample solution (1 µL) was injected into an RTX-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The oven temperature was initially maintained at 40 ºC and increased to 100 ºC at 15 ºC/min, followed by a ramp to 260 ºC at 4 ºC/min and held for 1 min. The injector temperature was maintained at 250 ºC, while ion source and interface temperatures were set at 230 ºC and 270 ºC, respectively. Helium (99.99% purity) was used as the carrier gas at a constant flow rate of 1.0 mL/min. Mass spectra were recorded in the range of 40-650 *m/z*. Compound identification was performed by comparing the obtained spectra with NIST20 and WILEY spectral libraries along with retention characteristics. Relative abundance of compounds was calculated from the normalized chromatographic peak areas. No derivatization procedure was employed prior to analysis; therefore, the method primarily detected volatile and semi-volatile constituents of the extract, while highly polar or non-volatile compounds may not have been efficiently represented [14].

Proximate analysis

Total protein content: An approximate quantity of 100 mg of *W. coagulans*, in powdered form, was combined with 2 mL of distilled water. Then, the solution was combined with 5 mL of 10% TCA solution to precipitate the proteins. The solution was centrifuged. The precipitated proteins were removed. Then, the precipitate was combined with 50 mL of deionized water. Approximately 0.4 mL of sample solution was used for the analysis. Working standards were prepared by mixing 1 mL of deionized water with varying volumes (0.2-1.0 mL) of the standard solution. Alkaline copper reagent (4.5 mL) was added to each tube, followed by incubation at room temperature. Subsequently, 0.5 mL of diluted Folin-Ciocalteu reagent was added and the mixture was incubated at room temperature for 15 min followed by 20 min in the dark. The absorbance was measured at 660 nm and the protein content was determined using a standard calibration curve. [15,16].

Total carbohydrate content: Carbohydrate content was estimated using the anthrone method [17]. Approximately 100 mg of *W. coagulans* fruit powder was dissolved in 100 mL of distilled water. A 0.5 mL sample was taken for the test. Standard glucose solutions with concentrations ranging from

100 to 500 µg/mL were prepared, then diluted to 1 mL with deionized water. Each tube was filled with 4 mL of fresh anthrone reagent and mixed well. The tubes were then placed in a boiling water bath for 10 min and subsequently allowed to cool to room temperature. At 660 nm, the resulting green colour was measured. A standard calibration curve was used to calculate the amount of carbohydrates contained.

Crude fibre content: Crude fibre content was determined by sequential acid and alkali digestion followed by drying and incineration [18]. Approximately 5 g of *W. coagulans* powder was boiled in 200 mL of 1.25% sulphuric acid, filtered through muslin cloth and washed with hot water. The residue was then boiled in 200 mL of 1.25% NaOH, filtered and washed until neutral. It was transferred to a pre-weighed silica crucible (W_1), dried at 130 ºC for 2 h, cooled and weighed (W_2). The residue was incinerated at 600 ºC for 30 min, cooled in a desiccator, and weighed again (W_3). The crude fibre content was calculated from the weight difference before and after incineration. Fibre content was calculated using the following formula:

$$\text{Fibre } (\%) = \frac{W_2 - W_3}{\text{Weight of sample}} \times 100$$

Moisture content: Approximately 2 g of *W. coagulans* fruit powder was taken in a pre-weighed petri dish, labelled W_1 and then dried for 4 h at 105 ºC in a hot-air oven. In a desiccator, the sample was chilled and weighed again, labelled W_2. The drying operation was repeated at 30 min intervals to constant weight. The percentage of weight loss was used to calculate the moisture content using the following formula [19]:

$$\text{Moisture content } (\%) = \frac{\text{Weight of sample} - (W_2 - W_1)}{\text{Weight of sample}} \times 100$$

Ash content: Approximately 5 g of *W. coagulans* fruits powder was placed in a pre-weighed silica crucible (W_1) and dried at 100 ºC for 2 h. The sample was then incinerated at 600 ºC for ~6 h or until a constant weight was achieved. After cooling in a desiccator, the crucible and remaining ash were weighed (W_2). Ash content was calculated as the percentage of residue remaining after incineration [20] using the following formula:

$$\text{Ash content } (\%) = \frac{W_1 - W_2}{\text{Weight of sample}} \times 100$$

Quantitative estimation of secondary metabolites

Total phenolic content (TPC): Total phenolic content (TPC) of the *W. coagulans* extract was determined using the Folin-Ciocalteu method [21]. The extract (5 mg) was dissolved in 10 mL of ethanol. The test samples and catechol standard (0.1-0.5 mL) were diluted with distilled water to a final volume of 3 mL. Folin-Ciocalteu reagent (0.5 mL) and 7% Na_2CO_3 solution (1 mL) were added to each mixture followed by incubation at room temperature for 10 min. The absorbance was measured at 640 nm and TPC was expressed as catechol equivalents per gram of extract.

Total flavonoid content (TFC): The flavonoid content was analyzed using an $AlCl_3$ colourimetric assay [22]. The extract (5 mg) was dissolved in 10 mL of ethanol. Aliquots of the sample (0.1, 0.3 and 0.5 mL) and quercetin standard (0.1-0.5 mL) were mixed with 4 mL of distilled water. Sodium nitrite (0.3 mL, 5%) was added and the mixture was allowed to react for 5 min. Aluminium chloride solution (0.3 mL, 10%) was then added and left for 6 min followed by 2 mL of 1 M NaOH. Absorbance was measured at 510 nm and total flavonoid content was expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g).

Nutritional composition

Estimation of α-tocopherol (vitamin E): α-Tocopherol content was determined using a modified colorimetric method [23]. Dried *W. coagulans* fruit powder (500 mg) was extracted with 10 mL of ethanol by shaking for 15 min. The mixture was centrifuged at 3000 rpm for 5 min and the supernatant was collected. Ferric chloride solution (0.2 mL) and 2,2′-dipyridyl solution (0.2 mL) were added and then 4 mL of butanol. After vigorous shaking to develop the colour, the absorbance was measured at 520 nm. α-Tocopherol content was estimated using an external standard calibration curve.

Estimation of potassium: Potassium content was determined by flame photometry following acid digestion [24]. Approximately 1 g of dried plant material was digested using 0.5 mL of sulphuric acid, 0.5 mL of perchloric acid and 15 mL of conc. nitric acid until a clear solution was obtained. After cooling, the digest was diluted with water, filtered through Whatman No. 2 filter paper and made up to 50 mL. Potassium concentration was measured using a flame photometer calibrated with standard potassium chloride solutions.

Estimation of calcium: Calcium content was determined using the *o*-cresolphthalein complexone method [25]. Approximately 2 g of powdered plant material was digested with a 2:1 (v/v) mixture of nitric and perchloric acids under controlled heating until a clear solution was obtained. After cooling, the digest was diluted with deionized water. An aliquot of the sample solution was mixed with 1 mL of *o*-cresolphthalein complexone reagent and 1 mL of AMP buffer followed by incubation at room temperature for 5 min. The absorbance was measured at 570 nm using a UV-visible spectrophotometer.

In vitro assay for antioxidant activity

DPPH assay: The antioxidant activity of *W. coagulans* extract was evaluated using the DPPH radical scavenging assay using ascorbic acid as the reference standard [26]. Different extract concentrations (100-500 µg/mL) were mixed with 1 mL of DPPH solution and incubated in the dark for 30 min. The absorbance was measured at 517 nm using a colorimeter. A DPPH solution without the extract served as the control.

$$\text{Scavenging } (\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100$$

Potassium ferricyanide reducing power assay: The reducing power of the ethanolic *W. coagulans* extract was evaluated using the potassium ferricyanide method [27]. Different extract concentrations (100-500 µg/mL) were adjusted to a final volume of 1 mL with solvent. Phosphate buffer (2.5 mL) and potassium ferricyanide solution (2.5 mL) were added to each tube, followed by incubation at 50 ºC for 20 min. Trichloroacetic acid (TCA) was then added and the mixtures were centrifuged at

3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of deionized water followed by the addition of ferric chloride solution and then absorbance was measured at 700 nm.

Calcium oxalate crystallization-Inhibitory activity

Nucleation assay: The CaOx nucleation inhibition was estimated using a method adapted from Bawari *et al.* [28]. Calcium chloride (5 mmol/L) and sodium oxalate (7.5 mmol/L) solutions were prepared in 0.05 M Tris-HCl buffer containing 0.15 M NaCl at pH 6.5. The *W. coagulans* extract was diluted to concentrations of 100-500 µg/mL. Calcium chloride (3 mL) and sodium oxalate (3 mL) solutions were mixed with 1 mL of each extract concentration and incubated at 37 ºC for 30 min. In this method, Cystone was served as the positive control. The optical density was measured at 620 nm and the percentage inhibition of CaOx nucleation was calculated using the following formula:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}}\right) \times 100$$

Aggregation assay: CaOx crystal aggregation inhibition was evaluated using the method described by Rajeshwari *et al.* [29]. Calcium oxalate crystals were prepared by mixing equal volumes of 50 mmol/L calcium chloride and sodium oxalate solutions. The mixture was incubated at 60 ºC for 1 h followed by overnight incubation at 37 ºC. The resulting crystals were dried and resuspended in 0.05 M Tris-HCl buffer containing 0.15 M NaCl at pH 6.5 to obtain a final concentration of 0.8 mg/mL. Aliquots of the *W. coagulans* extract (100-500 µg/mL) were added to 3 mL of the crystal suspension, vortexed, and incubated at 37 °C for 30 min. The absorbance was measured at 620 nm and the percentage inhibition of CaOx crystal aggregation was calculated using the following formula:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}}\right) \times 100$$

Microscopic analysis: The morphological changes in CaOx crystals were examined using an optical microscope at 400× magnification and images were captured digitally. All experiments were performed in triplicate.

Anti-glycation assay: The anti-glycation activity of the *W. coagulans* extract was assessed using the method described by Premakumara & Abeysekera [30] with slight modifications. Fructose (1000 mM) and bovine serum albumin (20 mg/mL) were prepared in 200 mM phosphate buffer (pH 7.4). The reaction mixture contained 1.25 mL of BSA solution, 1 mL of fructose solution, different concentrations of the plant extract and phosphate buffer to a final volume of 5 mL. Aminoguanidine served as the positive control, while the control contained BSA and fructose without the extract. Blank sample containing the extract without BSA or fructose was used to calibre the fluorescence. The mixtures were incubated at 37 ºC for 7 days under sterile conditions. Advanced glycation end product (AGE) formation was monitored by measuring fluorescence at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. The percentage inhibition of glycation was calculated after blank correction using the following formula:

$$\text{Inhibition (\%)} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}}\right) \times 100$$

Statistical analysis: All experiments were independently performed in triplicate (n = 3) and the results are expressed as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism version 10.0 (GraphPad Software Inc., San Diego, USA). Comparisons between groups were performed using one-way or two-way analysis of variance (ANOVA), as appropriate, followed by Sidak's multiple comparison post hoc test. For assays in which IC_{50} was an applicable endpoint, IC_{50} values were determined using non-linear regression analysis based on a four-parameter logistic dose-response curve model. The goodness-of-fit of the regression models was evaluated using coefficient of determination (R^2) values. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical screening: Preliminary phytochemical screening of *W. coagulans* fruit extracts revealed differences in the composition of the chloroform, ethyl acetate and ethanolic extracts (Table-1). The ethanolic extract showed the widest phytochemical profile, with positive tests for alkaloids, carbohydrates, glycosides, flavonoids, phytosterols, phenolic compounds, cardiac glycosides and reducing sugars. The ethyl acetate extract contained alkaloids, carbohydrates, glycosides, phenolic compounds and phytosterols, whereas the chloroform extract showed the presence of flavonoids and phytosterols. Proteins, tannins, quinones and saponins were not detected in any of the extracts.

TABLE-1
PRELIMINARY PHYTOCHEMICAL SCREENING DATA OF *W. coagulans* FRUIT EXTRACTS OBTAINED USING DIFFERENT SOLVENTS

Phytochemical constituent	Chloroform	Ethyl acetate	Ethanol
Alkaloids	Absence	Presence	Presence
Carbohydrate	Absence	Presence	Presence
Glycosides	Absence	Presence	Presence
Proteins	Absence	Absence	Absence
Flavonoids	Presence	Absence	Presence
Phenols	Absence	Presence	Presence
Tannins	Absence	Absence	Absence
Phytosterol	Presence	Presence	Presence
Cardiac glycosides	Absence	Absence	Presence
Reducing sugar	Absence	Absence	Presence
Quinones	Absence	Absence	Absence
Saponins	Absence	Absence	Absence

Proximate analysis of functional food: Proximate composition of dried *W. coagulans* fruit powder is given in Table-2. The dried fruit powder contained appreciable amounts of carbohydrates (60 ± 0.57%), dietary fibre (16.45 ± 0.10%), along with lower proportions of protein (5.2 ± 1.41%), ash (4.4 ± 0.02%) and moisture content (7.7 ± 0.14%).

The wider range of phytochemical classes detected in the ethanolic extract may reflect the ability of ethanol to extract compounds with different polarities. This finding is consistent with previous reports of diverse bioactive constituents in ethanolic and hydroalcoholic extracts of *W. coagulans* [10].

TABLE-2
PROXIMATE COMPOSITION OF DRIED *W. coagulans* FRUIT POWDER

Nutrient composition	Quantity
Moisture	7.70 ± 0.1400
Ash	4.40 ± 0.0200
Fibre content	16.45 ± 0.1000
Carbohydrate	60.00 ± 0.5773
Protein	5.20 ± 1.4191

Values are expressed as mean ± standard deviation (SD) of triplicate determinations. All parameters are presented in percentage (% w/w) except where otherwise specified.

The plant is also known to contain withanolides and other compounds associated with reported antidiabetic, antioxidant, anti-inflammatory and neuroprotective activities [11]. The present screening results indicate that the choice of solvent influenced the qualitative phytochemical profile of the fruit extracts.

The dried *W. coagulans* fruit powder contained 60 ± 0.57% carbohydrates and 5.2 ± 1.4191% protein. Carbohydrates constituted the major fraction of the measured nutritional composition, while protein accounted for a smaller proportion. The relatively low moisture content may favour storage stability by reducing conditions beneficial to microbial growth. Ash content represents the inorganic residue remaining after incineration, while the measured lipid and fibre fractions contribute to the proximate composition of the fruit material. Previous studies have reported glycosides, withanolides and other steroidal compounds in *W. coagulans* [12,31]. The high carbohydrate content observed in the present study may be associated with sugars and other carbohydrate related constituents.

Quantitative estimation of secondary metabolites: The quantitative analysis of the ethanolic fruit extract of W. coagulans revealed a total phenolic content (TPC) of 75.6 mg catechol equivalents/g extract and a total flavonoid content (TFC) of 60 mg quercetin equivalents/g extract (Table-3). The measured phenolic and flavonoid contents are consistent with previous reports of these compounds in polar-solvent extracts of *W. coagulans* [32]. Differences between studies may be due the extraction procedure, solvent composition, geographical origin, growing conditions and fruit maturity.

TABLE-3
QUANTITATIVE ESTIMATION OF SECONDARY METABOLITES IN *Withania coagulans* FRUIT EXTRACT

Phytochemical constituent	Quantity (mg/g)
Total phenolic content (mg catechol equivalents/g)	75.6
Total flavonoid content (mg quercetin equivalents/g)	60.0

Values represent mean ± SD (n = 3).

Nutritional composition: The nutritional composition of dried *W. coagulans* fruit powder is shown in Table-4. The dried fruit material contained measurable amounts of α-tocopherol (822.31 µg/100 g), potassium (372.54 mg/100 g) and calcium (289.85 mg/100 g), which confirm the presence of nutrient relevant micronutrients. The presence of α-tocopherol contributes to the nutritional profile of the dried fruit material.

TABLE-4
NUTRITIONAL COMPOSITION OF DRIED *W. coagulans* FRUIT

Nutrient	Quantity
α-Tocopherol	822.31 µg/100 g
Potassium	372.54 mg/100 g
Calcium	289.85 mg/100 g

Values represent mean ± SD (n = 3).

The dried *W. coagulans* fruit material contained measurable amounts of potassium, calcium and α-tocopherol, which contribute to its nutritional profile. These findings are consistent with previous reports of nutritionally relevant constituents in *W. coagulans* [10].

Gas chromatography-mass spectroscopy (GC-MS) analysis: GC-MS analysis of the *W. coagulans* fruit extract resulted in the identification of 55 compounds based on the spectral library matched within a retention time range of 7.747-59.827 min (Fig. 1). The major constituents identified based on peak area normalization are *trans*-9,12-octadecadienoic acid propyl ester (21.53%), (Z)-ethyl heptadec-9-enoate (15.03%), 9-octadecenal (Z)- (13.00%), linoelaidic acid (7.94%) and hexadecanoic acid ethyl ester (6.94%) (Table-5). Based on GC-MS analysis, the chemical profile was dominated by fatty acid esters and unsaturated lipid derivatives, accompanied by aldehydes and smaller proportions of phenolics, ketones, hydrocarbons and terpenoid-related compounds. The lipid associated compounds accounted for the largest fraction of the detected constituents. Minor compounds may contribute to the extract's biological activity, although their individual effects cannot be established from the compositional data alone. It should be noted that no withanolides were detected in the present analysis. Their absence from the chromatographic profile does not confirm their absence from the extract, as these non-volatile, thermolabile steroidal lactones are not detected by direct GC-MS without suitable derivatisation. The chemical profile is consistent with previous reports of diverse phytoconstituents in *W. coagulans* extracts [33].

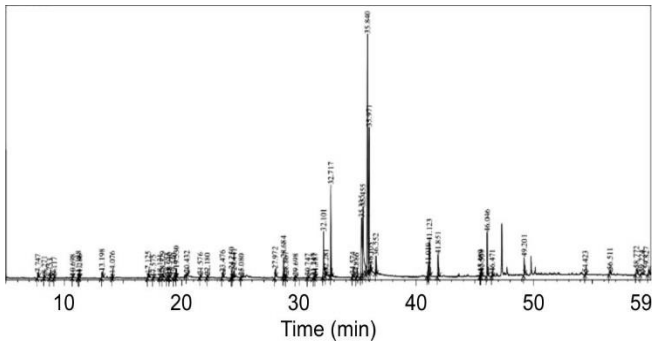


Fig. 1. GC-MS chromatogram of *W. coagulans* fruit extract

Antioxidant activity: The antioxidant potential of ethanolic fruit extract of *W. coagulans* was assessed by using DPPH radical scavenging assay and the results are shown in Fig. 2. The extract exhibited a dose dependent increase in the radical scavenging activity, which was found to be 37.98% at 100 µg/mL and 74.00% at 500 µg/mL. Ascorbic acid used as reference standard showed comparatively higher antioxidant

TABLE-5
LIST OF MAJOR PHYTOCHEMICAL COMPOUNDS IDENTIFIED IN *W. coagulans* FRUIT EXTRACT BY GC-MS ANALYSIS

Peak	Retention time (min)	Area	Area (%)	Similarity	Name
39	35.840	12948444	21.53	93	*trans,trans*-9,12-Octadecadienoic acid, propyl ester
40	35.971	9040310	15.03	87	(Z)-Ethyl heptadic-9-enoate
38	35.455	7817958	13.00	90	9-Octadecenal, (Z)-
37	35.335	4774660	7.94	95	Linoelaidic acid
34	32.717	4175830	6.94	96	Hexadecanoic acid, ethyl ester
32	32.101	2906870	4.83	94	Pentadecanoic acid
44	41.123	2508522	4.17	90	(E)-9-Octadecenoic acid ethyl ester
48	46.046	2305293	3.83	94	Docosanoic acid, ethyl ester
45	41.851	1490834	2.48	95	Eicosanoic acid, ethyl ester
42	36.552	937502	1.56	92	Heptadecanoic acid, ethyl ester

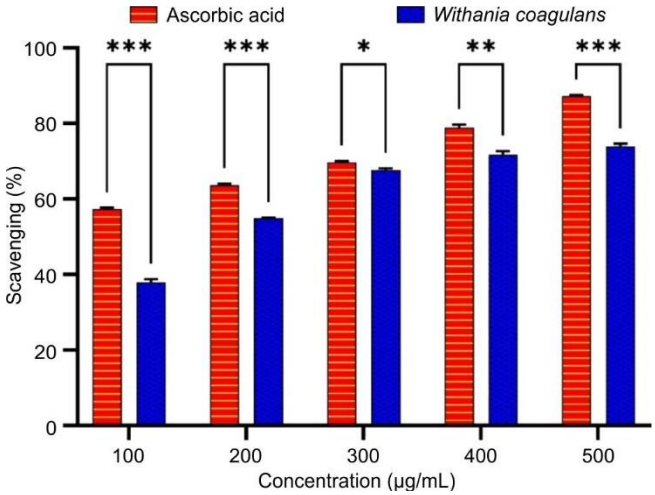


Fig. 2. DPPH radical scavenging activity of *W. coagulans* fruit extract

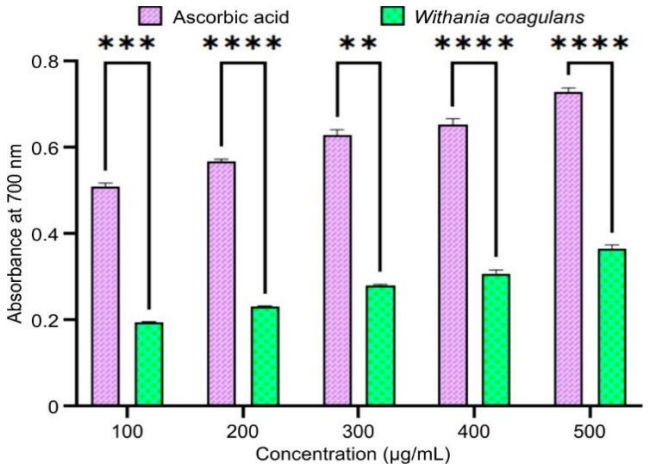


Fig. 3. Potassium ferricyanide reducing power of *W. coagulans* ethanolic fruit extract and ascorbic acid at 100-500 µg/mL. Absorbance was measured at 700 nm. Values are mean ± SD (n = 3)

activity within the tested concentration range. The extract showed a moderate potential in scavenging free radicals with an IC_{50} value of 170.8 µg/mL.

The ethanolic extract of *W. coagulans* fruit exhibited concentration-dependent DPPH radical-scavenging activity, although the activity was lower than that reported for some other extracts of the plant. Similar concentration-dependent responses have been described in previous studies [1]. A leaf extract reportedly had an IC_{50} value of approximately 147.4 µg/mL, which suggest a stronger radical-scavenging activity than that of the fruit extract examined in this study. The methanolic extracts have also shown up to 90% radical scavenging at higher concentrations, alongside reported total phenolic and flavonoid contents of 71.53 mg GAE/g and 64.32 mg QE/g, respectively [34]. Differences between these findings may be related to the plant part, solvent system and extraction conditions. Although phenolics and flavonoids were detected in the present extract, their individual contributions to DPPH scavenging were not established.

The reducing power of the ethanolic fruit extract was assessed using the potassium ferricyanide assay, with absorbance measured at 700 nm (Fig. 3). The absorbance increased from 0.1935 ± 0.0022 at 100 µg/mL to 0.3646 ± 0.0093 at 500 µg/mL. The reference standard ascorbic acid showed higher absorbance values across the tested concentration range with the values increasing from 0.5081 ± 0.0092 to 0.7300 ± 0.0085. The concentration-dependent increase in absorbance indicates that the extract can reduce ferric ions under the assay conditions, although its reducing capacity was lower than that of ascorbic acid. Similar concentration-related increases in reducing power have been reported for *W. coagulans* extracts [10]. The observed activity is consistent with the presence of antioxidant constituents, but the contribution of individual compounds remains unverified.

Calcium oxalate crystallization-Inhibitory activity: The ethanolic fruit extract of *W. coagulans* inhibited calcium oxalate (CaOx) crystal nucleation in a concentration-dependent manner (Fig. 4), with inhibition increasing from 17.06% at 100 µg/mL to 85.03% at 500 µg/mL. The calculated IC_{50} was 279 µg/mL, while cystone exhibited stronger inhibitory activity across the tested concentration range. A similar trend was observed in the crystal aggregation assay (Fig. 5), where inhibition increased from 18.99% to 82.89% over the same concentration range, with an IC_{50} of 282 µg/mL. Cystone again showed higher activity than the extract. These findings indicate that the fruit extract interfered with CaOx nucleation and aggregation under the experimental conditions.

The observed inhibitory activity is consistent with the reports of CaOx crystallization inhibition by other plant derived compounds. Naikal *et al.* [35] reported inhibitory effects of quercetin and betulin isolated from *Aerva lanata*, while methanolic seed extracts of *Trigonella foenum-graecum* and *Nigella sativa* showed inhibition of CaOx formation at 5 mg/mL [36].

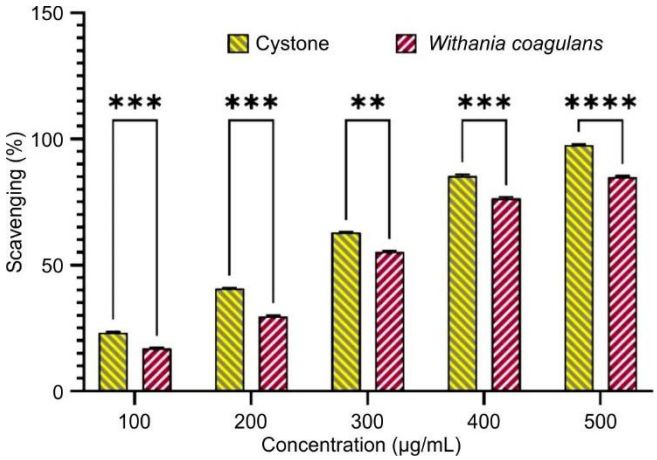


Fig. 4. Concentration-dependent inhibition of calcium oxalate nucleation by *W. coagulans* extract and cystone (mean ± SD, n = 3)

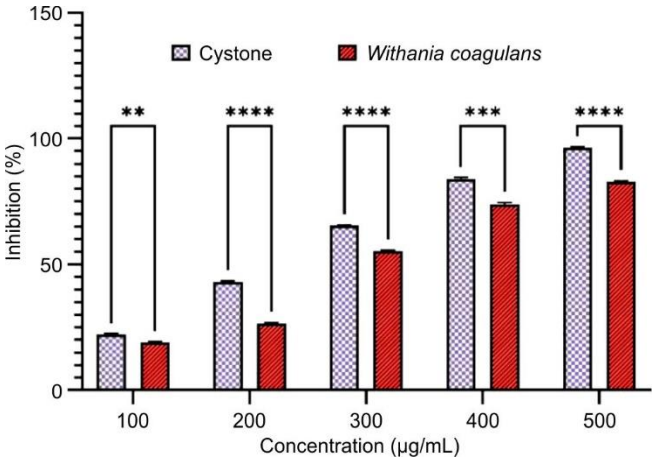


Fig. 5. Concentration-dependent inhibition of calcium oxalate crystal aggregation by *W. coagulans* extract and cystone (mean ± SD, n = 3)

Direct comparison is limited by differences in extract composition, test concentrations and experimental methods. The present results provide preliminary *in vitro* evidence of the potential of *W. coagulans* fruit extract to interfere with CaOx crystallization, although its activity was lower than that of cystone. A limitation of the study is the absence of extract only blanks to account for background absorbance or light scattering from the plant material. Such interference may

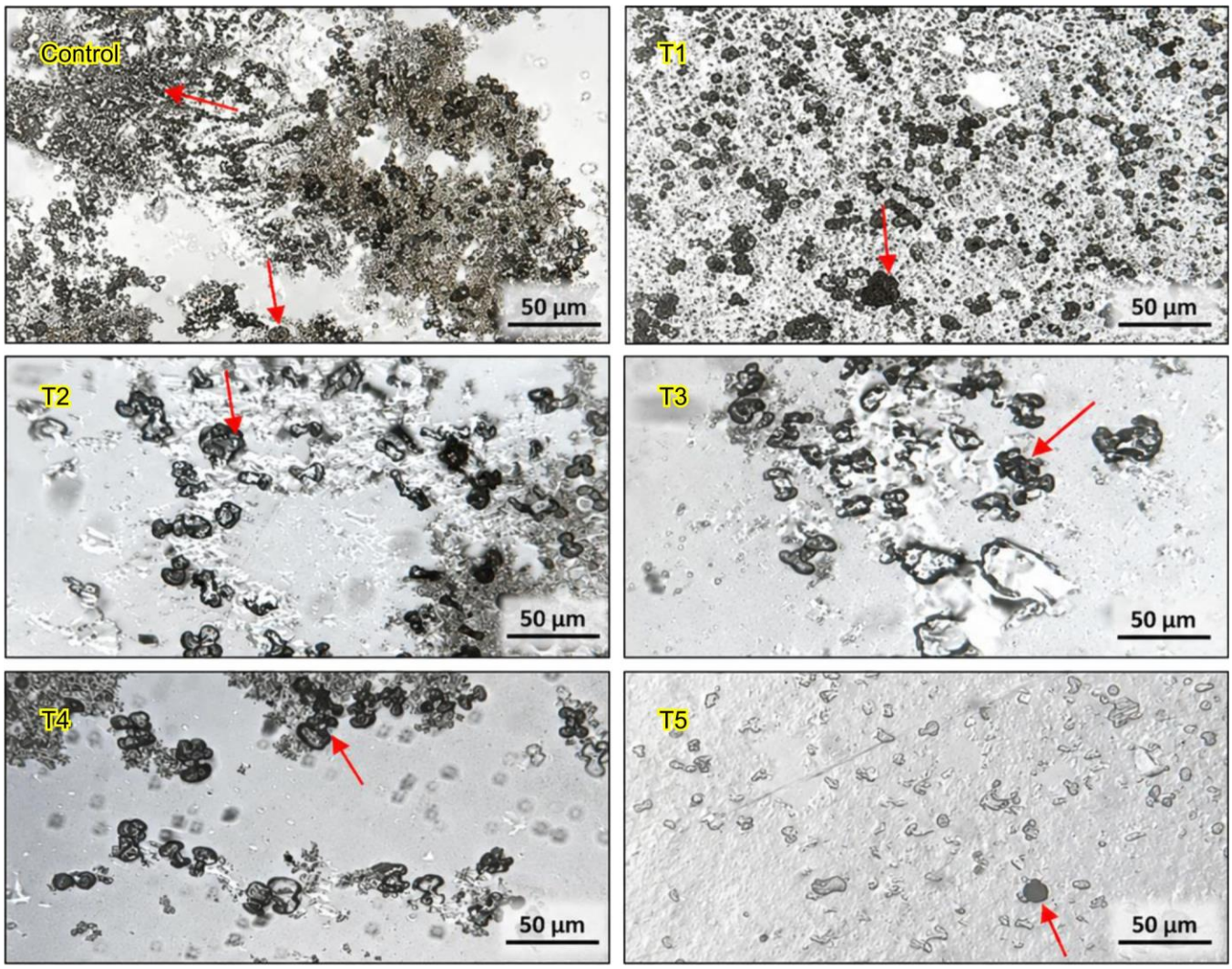


Fig. 6a. Microscopic analysis (400×) of calcium oxalate crystal nucleation showing reduced crystal size and number in *W. coagulans* treated groups compared to control. Arrows indicate crystal formation sites

affect the calculated inhibition values, particularly at higher extract concentrations.

Microscopic observation: Microscopic examination of CaOx crystals at 400× magnification revealed differences between the control and *W. coagulans*-treated groups (Fig. 6a-b). The control displayed numerous large, well-defined crystals arranged in dense clusters. In the treated groups (T1-T5), crystal size and apparent abundance decreased with increasing extract concentration and the crystals appeared more dispersed and less aggregated. These changes were particularly evident in T4 and T5, where crystal clusters were sparse or absent in some microscopic fields. The treated samples also contained crystals with irregular outlines and less distinct morphology than those in the control. These observations suggest that the extract may interfere with CaOx crystal formation and aggregation. The microscopic findings are consistent with the nucleation and aggregation assay results, supporting the inhibitory activity of *W. coagulans* fruit extract under the tested *in vitro* conditions.

Anti-glycation activity: The anti-glycation activity of the ethanolic extract of *W. coagulans* fruit was evaluated using the BSA-fructose glycation model (Fig. 7). The extract showed a concentration-dependent increase in glycation inhibition, although aminoguanidine used as the reference standard exhibited stronger activity across the tested concentration range. The extract had an IC_{50} value of 259 µg/mL, which indicates moderate inhibitory activity under the experimental conditions.

Oxidative stress can promote the formation of advanced glycation end products (AGEs) through glycoxidative reactions. Plant-derived phenolic compounds have been investigated for their ability to interfere with glycation reactions and AGE formation. Certain phenolics may affect the Maillard reaction pathway and reduce protein cross-linking, although the mechanism responsible for the activity of the present extract was not examined. The concentration-dependent inhibition observed is consistent with previous reports of plant polyphenolic extracts in BSA-sugar glycation models [37].

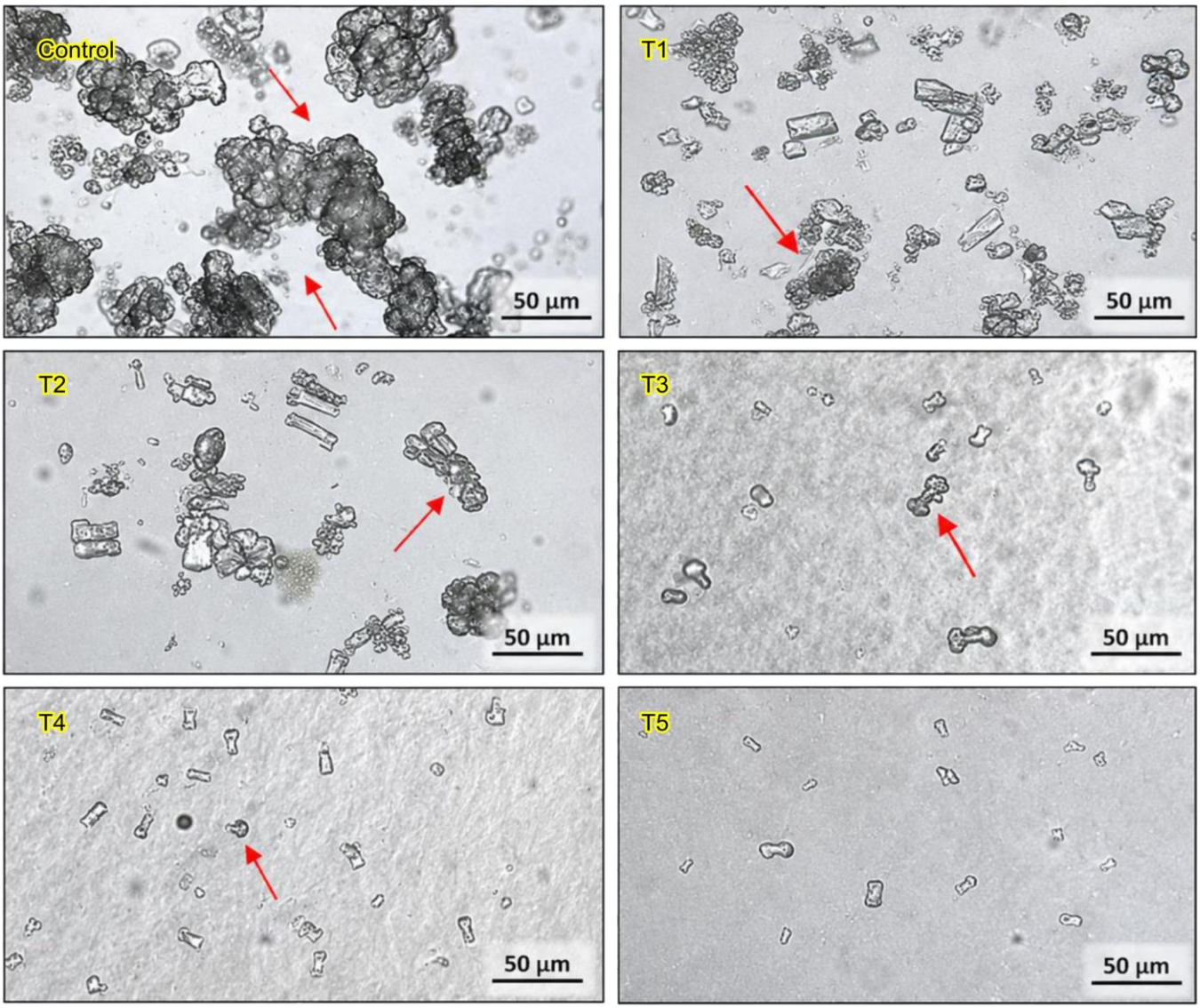


Fig. 6b. Microscopic analysis (400×) of calcium oxalate crystal aggregation showing reduced clustering and dispersion of crystals in treated groups compared to control. Arrows indicate aggregation sites

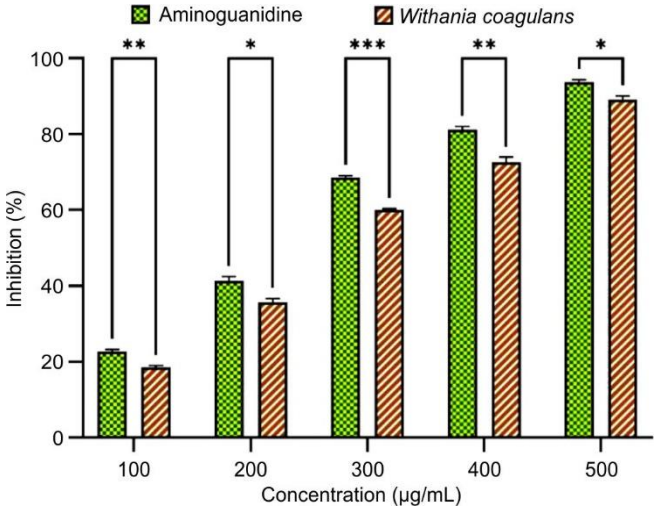


Fig. 7. Concentration-dependent inhibition of BSA glycation by *W. coagulans* extract and aminoguanidine (mean ± SD, n = 3)

Fructose- and methylglyoxal-induced glycation systems have likewise been used to evaluate natural compounds with aminoguanidine as a reference inhibitor [38]. These similarities support the suitability of the experimental model for assessing anti-glycation activity.

Conclusion

The ethanolic fruit extract of *W. coagulans* exhibited measurable phytochemical constituents along with moderate antioxidant and anti-glycation activities and inhibitory effects on calcium oxalate (CaOx) nucleation and aggregation under the tested *in vitro* conditions. The extract contained phenolic and flavonoid compounds, while the dried fruit material showed a nutritionally relevant composition. GC-MS profiling provided a preliminary chemical profile dominated by fatty acid derivatives and other lipophilic constituents, although the contribution of individual compounds to the observed biological activities remains unconfirmed. The inhibition of CaOx nucleation and aggregation suggests potential interference with crystal formation. The study was limited by the use of a crude extract and biochemical assays without cell-based validation or mechanistic investigations. The absence of extract-only background controls in the CaOx assays also leaves possible interference from absorbance and light scattering unresolved.

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CONFLICT OF INTEREST

The authors declare that there is no conflicts of interest regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language and no data, results or scientific conclusions were generated by AI. All analyses, interpretations and conclusions are the authors' own. The authors reviewed and edited the content and take full responsibility for the published work.

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