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Research Article

Thin Layer Chromatographic Analysis and Antioxidant Activities of Methanol Extract of Leaves of *Carica papaya* L.

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ABSTRACT

Traditional plants contain various secondary metabolites such as alkaloids, terpenoids, steroids and phenolic compounds which have shown antioxidant activities that include scavenging free radical species and inhibiting the production of reactive species resulting from normal cell metabolism. The present study was undertaken to analyse the thin layer chromatography and antioxidant activities of methanol extract of leaves of *Carica papaya*. The antioxidant activities were carried out by DPPH free radical scavenging assay, $OH^{\bullet}$ radical scavenging assay, $NO^{\bullet}$ radical scavenging assay, Fe^{3+} reducing power assay and phosphomolybdenum reduction assay.

Keywords: *Carica papaya*, DPPH assay, secondary metabolites, antioxidant, IC_{50}.

INTRODUCTION

Medicinal plants have great importance in formulation of medicines due to enriched quality of phytochemicals produced by them as the byproduct of secondary metabolism. Antioxidants play important role in body defense system against reactive oxygen species (ROS) as they combine with reactive oxygen species and null their toxic effect [1]. Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide and other exogenous factors are generally the cause of several fatal diseases such as coronary heart disease, stroke, rheumatoid arthritis, diabetes and cancer [2]. Thus, any plant possessing antioxidant activity could be a potential lead for curing any of the above ailments.

Carica papaya L. belongs to the small family Caricaceae and is one of the non-seasonal and evergreen fruits in Malaysia. The tree is always with a soft main trunk and tufted leaves at the top. Papaya fruits vary in size, shape, color and taste. In Malaysia, the preference is for the red fleshed varieties namely Eksotika. The skin color of papaya is usually green when immature, changing to fully reddish-orange when fully ripened. The changed color of the skin of fruit is an indicator of ripeness, and this change is considered mainly due to an increase in the carotene content and a decrease in chlorophyll. The color of papaya fruit flesh is determined largely by the presence of carotenoid pigments [3]. The red-fleshed papaya has 63.5% of total carotenoids as lycopene which is absent in yellow-fleshed fruit [4]. Papaya contains a broad spectrum of phytochemicals including enzymes (in the latex), carotenoids (in fruits and seeds), alkaloids (in leaves), phenolics (in fruits, leaves, and shoots), and glucosinolates (in seeds and fruits). Previous studies have shown that papaya possesses antihelmintic, antiprotozoan, antibacterial, antifungal, antiviral, anti-inflammatory, antihypertensive, hypoglycemic and hypolipidemic, wound healing, antitumor, free-radical scavenging, antisickling, neuroprotective, diuretic, abortifacient, and antifertility activities [5]. In Jamaica, the ripe papaya is used as topical ulcer dressings to promote granulation, healing, and reducing odor in chronic skin ulcers [6]. The green papaya fruits are used in Nigeria for treatment of diabetes mellitus,

hypertension, intestinal helminthiasis, jaundice and malaria and the leaves are used in India for abortion and treatment of asthma, beriberi, colic and fever[7]. The people living on the Gold Coast of Australia consumed the leaf juice for its purported anti-cancer activity [8]. The milky juice (latex) is employed as styptic when applied as external applications to burns. The objectives of the present study were to analyse thin layer chromatography and antioxidant activities of methanol extract of leaves of *Carica papaya*.

MATERIAL AND METHODS

Collection and authentication of plant material: Leaves of *Carica papaya* were collected from Porur, Chennai, India. The plant was authenticated by Prof. Dr. N. Raaman Centre for Advanced Studies in Botany, University of Madras, Chennai, India. Leaves were thoroughly washed and dried in shade for 10 d. Dried leaves were made into coarse powder using mechanical blender and stored in air tight container till further use.

Extract preparation: The leaves were ground and powdered material (500 g) was extracted using methanol as a solvent by maceration method. Initially, coarse powder of leaves was soaked in methanol for 72 h. Then the supernatant was filtered through filter paper. Powdered extracts were concentrated using rotary evaporator and a greenish-black coloured sticky residue was obtained.

Thin layer chromatography: Thin layer chromatography (TLC) was carried out for methanol extract of leaves of *C. papaya* on Merck TLC aluminium sheets, Silica gel 60 F_{254} (20 x 20 cm), precoated plates. The methanol extract of leaves of *C. papaya* was spotted at 0.3 mm above from the bottom of the TLC plate. The chromatogram was developed in a mixture of suitable solvent system. The spots were visualized with UV light at 254 nm [9]. The R_f values of the coloured spots were recorded [10]

$$\text{\% of DPPH radical inhibition} = \frac{\text{Distance travelled by solute front (in cm)}}{\text{Distance travelled by the solvent front (in cm)}}$$

Phytochemical screening: The methanol extract of leaves of *C. papaya* was subjected to preliminary phytochemical screening using standard methods [11]. The methanol extract of leaves of *C. papaya* was screened for different classes of phytoconstituents such as alkaloids, steroids, terpenoids, glycosides, flavonoids, tannins, phenolic compounds, saponins and carbohydrates using specific standard reagents [12, 13].

Determination of total phenolic content (TPC): Phenolic content of methanol extract of leaves of *C. papaya* was determined by the Folin-Ciocalteu reagent method [14]. One mL of extract was mixed with 1 mL of Folin-Ciocalteu reagent (1:10 diluted) and 1 mL of sodium carbonate solution (7.5%). The samples were thoroughly mixed and stored in the dark for 30 min. Absorbance of the blue colored mixture was recorded at 765 nm against a blank containing the Folin-Ciocalteu reagent and sodium carbonate solution. The phenolic content expressed as gallic acid equivalent.

Determination of total flavonoid content: Total flavonoid content was determined by aluminium chloride ($AlCl_3$) method [15] using quercetin as a standard. The plant extract (0.1 mL) was added to 0.3 mL distilled water followed by 5% $NaNO_2$ (0.03 mL). After 5 min at 25°C, $AlCl_3$ (0.03 mL, 10%) was added. After another 5 min, the reaction mixture was treated with 0.2 mL of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 mL with water and the absorbance was measured at 510 nm. The result was expressed as quercetin equivalent.

DPPH radical scavenging assay: The antioxidant activity of methanol extract of leaves of *C. papaya* was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhyorazyl (DPPH) free radical according to the method described by Brand-Williams et al. [16] with slight modifications. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of plant extract solution of varying concentrations (100-600 µg/mL). Ascorbic acid was used as reference standard. Mixer of 1 mL methanol and 1 mL DPPH solution was used as control. The decrease in absorbance was measured at 517 nm after 30 min in dark using UV-Vis spectrophotometer. The percentage of DPPH radical inhibition was calculated as

$$\text{\% of DPPH radical inhibition} = \frac{\text{Control-Sample}}{\text{Control}} \times 100$$

ABTS + radical scavenging assay: The antioxidant capacity was estimated in terms of the ABTS + radical scavenging activity following the procedure described by Delgado-Andrade et al. [17]. Briefly, ABTS was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12 - 16 h before use. The ABTS

solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance of 0.70±0.02 at 730 nm. After the addition of 1 mL of plant extract solution of varying concentrations (10 - 60 µg/mL) to 1 mL of diluted ABTS $^{+}$ solution, the absorbance was measured after 30 min. The ABTS $^{+}$ radical-scavenging activity of the samples was expressed as

$$\% \text{ of ABTS}^{+} \text{ radical inhibition} = \frac{\text{Control-Sample}}{\text{Control}} \times 100$$

Hydroxyl (OH•) radical scavenging assay: Hydroxyl radical scavenging assay was measured according to the method of Klenin et al. [18]. Various concentrations (10-60 µg/mL) of methanol extract of leaves of *C. papaya* were added with 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1 mL of DMSO (0.85% v/v) in 0.1 M phosphate buffer, pH 7.4. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 90°C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1 mL of ice cold TCA (17.5% w/v). Three mL of Nash reagent (75 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone were mixed and made up to 1 L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of color formed was measured spectrophotometrically at 412 nm against reagent blank. The percentage of hydroxyl radical scavenging activity was calculated by the following formula

$$\% \text{ of OH radical inhibition} = \frac{\text{Control-Sample}}{\text{Control}} \times 100$$

Nitric oxide (NO•) radical scavenging assay: Different concentrations of 1 mL methanol extract of leaves of *C. papaya* with nitric oxide were assessed by the nitrite detection method as described by Sreejayan and Rao [19]. Nitric oxide was generated from sodium nitroprusside previously bubbled with nitrogen and measured by the Greiss reaction. To this, 0.25 mL of sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) was mixed with 0.25 mL of different concentrations (10–60 µg/mL) of extracts dissolved in the suitable solvent system and incubated at 30°C in the dark for 150 min. The control was run as above but the sample was replaced with the same amount of water. After the incubation period, 0.25 mL of Griess reagent A (1% sulphanilamide in 5% phosphoric acid) was added, and kept at 30°C for 10 min. After incubation, 0.25 mL of Griess reagent B (0.1% *N*-1-naphthylethylene diamine dihydrochloride) was added, mixed and incubated at 30°C for 20 min. The absorbance of the chromophore formed was read at 546 nm. The percentage of NO radical scavenging activity was calculated using the following formula

$$\% \text{ of NO radical inhibition} = \frac{\text{Control-Sample}}{\text{Control}} \times 100$$

Ferric (Fe^{3+}) reducing power assay: The reducing power assay of methanol extract of leaves of *C. papaya* was determined according to the method of Yen and Chen [20]. One mL each of varying concentrations of methanol extract solution (100-600 µg/mL) were mixed with 1 mL of 0.2 M phosphate buffer, pH 6.6, and 1% solution of potassium ferricyanide. The mixture was incubated at 50°C for 20 min, during which period ferricyanide was reduced to ferrocyanide. Then, 1 mL of 1% trichloroacetic acid was added to the mixture, which was then centrifuged at 5,000 *g* for 10 min. The upper layer of the solution was mixed with distilled water and 0.1% $FeCl_3$ and the absorbance at 700 nm was measured to determine the amount of ferric ferrocyanide (prussian blue) formed. Increased absorbance of the reaction mixture indicates the increase in reduction of ferricyanide.

Phosphomolybdenum reduction assay: The antioxidant capacity of the methanol extract of leaves of *C. papaya* was assessed as described by Prieto et al [21]. The methanol extract in dilution from 100-600 µg/mL was combined with reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM). The reaction mixture was incubated in a water bath at 90°C for 90 min. The absorbance of the coloured complex was measured at 695 nm. Ascorbic acid was used as standard reference. Increased absorbance of the reaction mixture indicates the increase in phosphomolybdenum reduction.

RESULTS AND DISCUSSION

Thin layer chromatography:

TLC analysis was carried out for methanol extract of leaves of *C. papaya* by using the solvent system of chloroform: methanol with the ratio of 9:1. The separated bands were visualized by UV light at 254 nm. The R_f values of the separated compounds were measured (Table 1 and Figure 1).

Phytochemical screening:

The phytochemical analysis was carried out according to the standard procedures. The results showed that the presence of terpenoids, alkaloids, flavanoids, phenols, steroids, glycosides,

carbohydrates and saponins in the methanol extract of leaves of *C. papaya* (Table 2).

Estimations of phenols and flavonoids:

Plant phenolics present in the fruits and vegetables have received considerable attention because of their potential biological activity. The diverse biological activities including anti-atherosclerotic, anti-carcinogenic and anti-inflammatory activities possessed by phenolic compounds such as flavonoids, phenolic acid, and tannins might be due to their antioxidant activity [22]. The yield of total phenolics and flavonoid content of methanol extract of leaves of *C. papaya* were 210.6 and 140.61 mg/g of extract, respectively (Table 3).

DPPH radical scavenging assay:

The DPPH is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by antioxidant [23]. It has been widely used to test the ability of compounds as free radical scavengers by hydrogen donors and to evaluate the antioxidant [24, 25]. The methanol extract of leaves of *C. papaya* exhibited a significant inhibition of DPPH activity. The percentage of inhibition was found to be 78.86 at 600 µg/mL (Table 4). It was compared with the standard ascorbic acid (Table 8). The scavenging ability of methanol extract of leaves of *C. papaya* was concentration dependent and expressed as IC_{50} values. Concentration of the sample necessary to decrease the initial concentration of DPPH• by 50% (IC_{50}) under the experimental condition was calculated. Therefore, a lower IC_{50} value indicates a higher antioxidant activity. The IC_{50} of DPPH radical scavenging activity was 104.71 µg/ mL concentration.

$ABTS^{•+}$ radical scavenging assay:

The $ABTS^{•+}$ radical cation scavenging activity also reflects hydrogen donating ability. Hagerman et al. [26] reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals (ABTS•+). Since, the extract from *C. papaya* has the ability to scavenge free radicals, thereby preventing lipid oxidation via a chain breaking reaction, they could serve as potential nutraceutical. The methanol extract of leaves of *C. papaya* exhibited a significant dose dependent inhibition of ABTS•+ radical cation scavenging activity. The percentage of inhibition was found to be 87.77 at 60 µg/mL concentration (Table 5). Ascorbic acid was used as the standard reference (Table 8). The IC_{50} of $ABTS^{•+}$ radical scavenging activity was 18.00 µg/ mL concentration.

Hydroxyl radical (OH•) scavenging assay:

Hydroxyl radical can be formed by the Fenton reaction in the presence of reduced transition metals (such as Fe^{2+}) and H_2O_2, which is known to be the most reactive of all the reduced forms of dioxygen and is thought to initiate cell damage by *in vivo* [27]. Scavenging of hydroxyl radical is an important antioxidant activity because of very high reactivity of the OH radical, enabling it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids, and nucleotides. Thus, removing OH• is very important for the protection of living systems. The hydroxyl radical scavenging potential of methanol extract of leaves of *C. papaya* was 53.80% at 60 µg/mL concentration (Table 6). The methanol extract of leaves of *C. papaya* showed hydroxyl radical scavenging activity which was increased with increasing concentration of extract and it was compared with the standard ascorbic acid (Table 8). The IC_{50} of $OH^{•}$ radical scavenging activity was 55.76 µg/ mL concentration.

Nitric oxide radical (NO•) scavenging assay:

Nitric oxide is an unstable free radical involved in many biological processes which is associated with several diseases. It reacts with oxygen to produce stable product nitrate and nitrite through intermediates and high concentration of nitric oxide can be toxic and inhibition of over production is an important goal [28]. Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as inhibition of platelet aggregation, neuronal signaling, regulation of cell mediated toxicity and smooth muscle relaxation. At physiological pH, sodium nitroprusside on mixing with aqueous solution generates nitric oxide. The nitric oxide reacts with oxygen to produce nitrite ions which can be estimated by using Greiss reagent. Nitric oxide scavengers compete with oxygen which results in less production of nitrite ions. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-(1- naphthyl) ethylenediamine dihydrochloride) was measured spectrophotometrically at 546 nm. The methanol extract of leaves of *C. papaya* exhibited a significant dose dependent inhibition of OH• radical scavenging activity. The percentage of inhibition was found to be 72.91 at 60 µg/mL (Table 6). Ascorbic acid was used as the standard reference (Table 8). The IC_{50} of $NO^{•}$ radical scavenging activity was 48.73 µg/ mL concentration.

Phosphomolybdenum reduction assay:

To evaluate the antioxidant capacity of extracts, this assay has been routinely used [29]. The antioxidant

capacity of the methanol extract of leaves of *C. papaya* was determined by the formation of green phosphomolybdenum complex, which was measured by the intensity of absorbance at maximum of 600 μg/mL concentration (absorbance 1.139) at 95°C (Table 7). During this process, the antioxidant compounds reduce Mo (VI) to Mo (V) and green phosphate/Mo (V) complex was formed with the maximal absorption at 695 nm. Ascorbic acid was used as the standard reference (Table 9).

Ferric (Fe^{3+}) reducing power assay:
Antioxidants can be explained as reductants, and inactivators of oxidants [30]. Some previous studies have also reported that the reducing power may serve as a significant indicator of potential antioxidant activity [31]. Antioxidant activity has been proposed to be related to reducing power. The methanol extract of leaves of *C. papaya* was used to investigate Fe^{3+} - Fe^{2+} transition to measure the reducing capacity of methanol extract of leaves of *C. papaya*. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Various mechanisms such as binding of transition metal ion catalysts, decomposition of peroxides, prevention of chain initiation, prevention of continued hydrogen abstraction and radical scavenging are attributed to the antioxidant activities of the antioxidants. The reducing power of methanol extract of leaves of *C. papaya* was measured at 600 μg/mL concentration (absorbance 0.246) and it exhibited a dose dependent reducing power activity. Ascorbic acid was used as the standard reference (Table 9).

It has been shown that the juice of leaves of *C. papaya* increased the platelet count in dengue patients [32], proteases from *Carica candamarcensis* (*V. cundinamarcensis*) exhibited antitumoral activity in Murine Melanoma B16F1 [33], methanol extract of leaves of *C. papaya* showed anti-fertilility in male wistar rats [34] and ethyl acetate crude extract of *C. papaya* leaves had antiplasmodial activity against *Plasmodium falciparum* [35]. Antioxidant and haemopoietic properties of leaves of *C. papaya* in the bone marrow [36] and hypoglycemic activity of aqueous extract of leaves of *C. papaya* have been established [37]. It has been proved that silver nanoparticles of leaves of *C. papaya* showed antibacterial activity against uropathogens such as *E.coli, Klebsiella pneumoniae* and *P. aeruginosa* [38]. Certainly, *C. papaya* with multitude of uses is an ethnomedicinal boon [39].

CONCLUSION
The methanol extract of leaves of *C. papaya* showed the presence of significant amount of phenols and flavonoids. Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity. The phenolic compounds may contribute directly to antioxidative action. In humans, from a diet rich in fruits and vegetables sources ingested daily, the polyphenolic compounds may exhibit inhibitory effects on mutagenesis and carcinogenesis. The present study clearly revealed the good antioxidant activity of leaves of *C. papaya* and thus the leaves of *C. papaya* have the potential to cure several diseases.

Table 1
R_f values of compounds from methanol extract of leaves of *C. papya* separated by thin layer chromatography

Spots observed	R_f value (UV 254 nm)
1	0.32
2	0.43
3	0.67
4	0.78
5	0.83
6	0.89

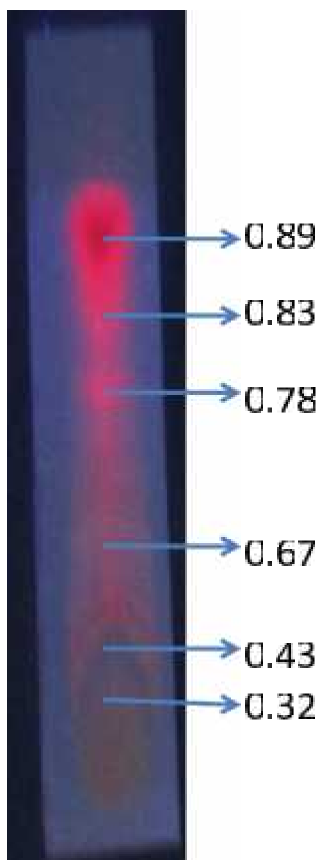

Figure 1
TLC of methanol extract of leaves of *C. papya*

Table 2
Qualitative phytochemical screening of methanol extract of leaves of *C. papya*

S. No	Phytoconstituents	Chemical reagents	Result
1.	Terpenoids	$CHCl_3$ + conc. H_2SO_4	+
2.	Alkaloids	Dragendorff's Reagent	+
3.	Flavanoids	NaOH solution	+
4.	Phenols	$FeCl_3$ solution	+
5.	Steroids	Acetic anhydride + conc. H_2SO_4	+
7.	Carbohydrates	-Naphthol alcohol + conc. H_2SO_4	+
8.	Saponins	Foam test	+

+ represents positive

Table 3
Quantity of phenols and flavonoids present in methanol extract of leaves of *C. papya*

S. No	Phytochemicals	Quantity (mg/g of extract)
1.	Phenols	210.60
2.	Flavanoids	140.61

Table 4
DPPH radical scavenging activity of methanol extract of leaves of *C. papaya*

S. No	Concentration (µg/mL)	% of inhibition
1	100	47.75±3.34
2	200	57.75±4.04
3	300	64.87±4.54
4	400	71.33±4.99
5	500	74.13±5.18
6	600	78.86±5.52

Table 5
$ABTS^{\cdot+}$ radical cation scavenging activity of methanol extract of leaves *C. papaya*

S. No	Concentration (µg/mL)	% of inhibition
1	10	23.33±.163
2	20	55.55±3.88
3	30	66.66±4.66
4	40	80.00±5.62
5	50	83.33±5.83
6	60	87.77±6.14

Table 6
Hydroxyl ($OH^{\bullet}$) and nitric oxide ($NO^{\bullet}$) radical scavenging activities of methanol extract of leaves of *C. papaya*

S. No	Concentration (µg/mL)	% of inhibition	
		$OH^{\bullet}$	$NO^{\bullet}$
1	10	16.8±1.17	15.20±1.06
2	20	22.6±1.58	33.33±2.33
3	30	23.8±1.66	38.80±2.71
4	40	24.1±1.68	45.10±3.15
5	50	35.7±2.49	51.30±3.59
6	60	53.8±3.76	72.91±5.10

Table 7
Ferric (Fe^{3+}) reducing power and phosphomolybdenum reduction assay of methanol extract of leaves of *C. papaya*

S. No	Concentration (µg/mL)	Fe^{3+} reducing Power	Phosphomolybdenum reduction
1	100	0.162±0.01	0.567±0.03
2	200	0.180±0.01	0.678±0.04
3	300	0.201±0.01	0.781±0.05
4	400	0.208±0.01	1.050±0.07
5	500	0.225±0.01	1.095±0.07
6	600	0.246±0.01	1.139±0.07

Table 8
DPPH, OH· and NO· radical scavenging activities of standard (Ascorbic acid)

S. No	Concentration (µg/mL)	DPPH	ABTS·+	OH·	NO·
1	2	03.34±0.23	25.00±1.75	82.00±5.74	37.14±2.59
2	4	13.88±0.27	32.25±2.25	86.04±6.02	40.00±2.81
3	6	21.59±1.51	43.54±3.04	89.14±6.23	48.57±3.39
4	8	37.25±2.60	37.9±2.65	96.89±6.78	54.28±3.79
5	10	70.62±4.94	51.61±3.61	92.24±6.45	60.00±4.23
6	12	70.95±4.96	52.41±3.66	97.67±6.83	62.85±4.39

Table 9
Ferric (Fe^{3+}) reducing power and phosphomolybdenum reduction of standard (Ascorbic acid)

S. No	Concentration (µg/mL)	Fe^{3+} reducing power	Phosphomolybdenum reduction
1	2	0.154±0.01	0.015±0.01
2	4	0.189±0.01	0.093±0.00
3	6	0.209±0.01	0.172±0.01
4	8	0.214±0.01	0.205±0.01
5	10	0.247±0.01	0.317±0.02
6	12	0.289±0.02	0.359±0.02

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