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Utilization of *Citrullus colocynthis* as Antibacterial Activity, Antioxidant and Food Preservation in Beef Luncheon Roll

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ABSTRACT
Citrullus colocynthus (CCE) consider as a promising medicinal fruit, which full of rich source of natural components. This study was investigated the effect of different extraction time of aqueous CME on antioxidants, antimicrobial, cytotoxicity components, and applied in luncheon. Results reveal that the aqueous CCE was rich in phenolic compounds, flavonoids, tannins, with higher antioxidants activity, besides has potential power against pathogenic bacteria and oxidative stress. Also, appeared high safety of CCE1 and CCE2 sample of CCE at the face of wi-38 and vero normal cell line. Nevertheless, CCE1&2 could be applied as food supplement in luncheon as antioxidant and food preservation from pathogenic bacteria .It has acceptable sensory evaluation similar to control luncheon sample.

***Keywords*:** *Citrullus colocynthus* (CCE) or (Citrus melon) aqueous CME, antioxidants, antimicrobial activity, sensory evaluation, wi-38 & vero normal cell line.

Introduction

The *Citrullus colocynthis* L. (bitter apple) is a desert plant and distributed in the Sahara, Egypt in Mediterranean, (Gurudeeban *et al.,* 2011; Jayaraman and Christina, 2013). The Phytochemical analysis of plant extracts revealed the presence of carbohydrate, protein, separated amino acid, tannins, saponins, phenolic, flavonoids, terpenoids, alkaloids, anthranol, steroids, Cucurbitacin A, B, C, D, E (α-elaterin), J, L, caffeic acid and cardic glycoloids (Alhajjaj *et al.,* 2010). The plant has also many using of traditional medicinal of digestive system diseases, diabetes, gastroenteritis, Constipation, dysentery and various bacterial infection. Also it used as antioxidant, analgesic, hypolipidemic, blood glucose-lowering effect, antimicrobial and anti-inflammatory affect. It has significant cytotoxic activity against human cancer cell lines of HL-60. The results from the present animal study showed that *C. colocyn* fruit, through its antioxidant and blood glucose-lowering activity, and a positive effect on the treatment of DN (Hassain *et al.,* 2014; Mohadesh *et al.,* 2020). *Citrullus colocynthis* extract for 7 day obviously reduced the impact of carbon tetrachloride toxicity on the serum markers of liver damage with ethyl acetate and chloroform and could protect liver against injury (Yangetal, 2013). CC natural, very cheap plants was used as adsorbent for phenol removal and it remove 70% of phenol per 30 min .It showed high affinity of waste ash adsorption which mainly composed of SiO_2 , Al_2O_3 and MgO (Mehdi *et al.,* 2018). It displayed analgesic at different doses without inducing acute toxicity. Topic results were showed using of CC immature followed by seeds, the stem and root extracts to possess appeared less significant inhibitory activity against analgesic and anti-inflammatory models respectively (Belsem *et al.,* 2010). CC form extracts types which was used in 36 studies were (ethanolic, aqueous, methanolic, hydroalco- holic, hydro-ethanol, chloroform, alkaloids, glycosidic, ethyl acetate, petroleum ether, saponosides, H_2O-methanol, and n-butanol), but the aqueous extract was the most frequent one. The *Citrullus colocynthis* dose was 300 mg/kg body weight/day most common of diabetes in 12 studies, Rats were the subject of almost all studies except eight of them, including rabbits (four studies), mice (one study), and dog (one study) (Mojtaba, *et al.* 2019). *Citrullus colocynthis* samples have inhibition effect on *Bacillus curuse*, *Staphylococcus aureus*, *Escherichia coli*, clostridium strains (Alhejjaj *et al.,* 2010)

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Materials and Methods

1. The *Citrullus colocynthus* extraction:

The *Citrullus colocynthus* (CC) fruit extracts were performed as follow:

Five hundred gram of bitter CC fruit were purchase from Egyptian local market and was cleaned perfectly from any dust or foreign substances. Fruits were washed with tap water and dried between two filter paper. Next seeds were isolated from fibers. After that, 560 ml distilled water were added to seeds contents and let stand overnight, then filtered using whatt man filter papers. The resultant filtrate is considered as sample CME1. The residual seeds and pulp covered with another 569 ml DW and boiled for 30 min, after cooling to the room temperature hot solution was filtered into clean flask and the filtrate consider as CME2. The residual from previous step re-boiled again with another 560 ml DW the filter into new clean flask and the fourth filtrate consider ad as CME3, CME4. Repeated the last treatment on CC fiber (F1, 2, 3, 4) According to Mojtaba *et al.* (2019).

2. Determination of cytotoxicity effect of C. colocynthis fruit extract using MTT assay

Human normal fetal lung cell line (Wi-38) and normal adult African green monkey kidney cell line (Vero) were used to investigate toxicity of C. colocynthis fruit extract according to method described by Mosmann (1983). Human Wi-38 and mammalian Vero cells were maintained in DMEM medium (Lonza, USA) containing 10% fetal bovine serum. These cell lines were subcultured for 2 weeks before assay using trypsin EDTA (Lonza, USA). Their viability and counting were detected by trypan blue stain and hemocytometer. Wi-38 and Vero were seeded in 96 well culture plate as 1x104 cells per well and incubated at 37ºC in 5% CO2 incubator. After 24h, cells were treated with the serial dilutions of CC (0, 6.25, 12.5, 25, 50 and 100 µg/ml). After 72 h incubation in 5% CO2 incubator, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated at 37ºC for 4h in 5% CO2 incubator. MTT (Sigma, USA) solution was removed and the insoluble blue formazan crystals trapped in cells were solubilized with 150 µl of 100% DMSO at 37ºC for 10 min. The absorbance of each well was measured with a microplate reader (BMG LabTech, Germany) at 570 nm. The safe dose (EC100) was estimated by the Graphpad Instat software as the concentration of C. colocynthis fruit extract at 100% cell viability.Additionally, morphological changes of C. colocynthis fruit extract treated normal human and mammalian cells were investigated in comparison with untreated control cells using phase contrast microscope supplemented with digital camera (Olympus, Japan).

3- Determination of free radical antioxidant activity by DPPH of CCE

Determination of the free radical scavenging activity by the 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Radical scavenging activities of various concentrations of C. colocynthis fruit extract were assayed according to Braca *et al.* (2001). All tested samples were incubated with DPPH (0.004% in methanol) in the dark for 30 min. Then the absorbance was measured at 517 nm. The percent of radical scavenging at each corresponding Log concentrations of each sample was used for calculating the IC50 value (50% inhibitory concentration) using GraphPad Instat software.

4. Antimicrobial Activity

4.1. Microorganisms and Culture Conditions

Pathogenic bacteria strains used were; *Bacillus cereus* EMCC 1006, *Staphylococcus aureus* EMCC 1351, *Escherichia* coli ATCC 25922 and *Clostridium perfringens.* All strains were obtained from Microbiological Resources Center (MERCIN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The strains were maintained by; the Department of Food Technology, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, Egypt in 60% glycerol/ LB culture at -80°C (Alhejjaj *et al.,* 2010; Hamad *et al.,* 2018).

4.2. Minimum Inhibitory Concentration (MIC) Determination

The bacterial strains were grown in nutrient broth at 37°C. A set of 7 concentration of reconstituted plant water extracts (100, 75, 50, 25, 12.5, 6.25 and 3.1 mg/ mL), were examined to determine the minimum inhibitory concentration (MIC) of each against a specific pathogenic strain (Hamad *et al.,* 2015). The zone of inhibition was calculated by measuring the diameter of the inhibition

zone around the well (mm), including the well diameter. The readings were taken in three different fixed directions in all triplicates and the average values were tabulated (Hamad *et al.,* 2018).

5- Determination of Total Phenolic Content in *Citrullus Colocynthis* Extract

The total phenolic content of the *Citrullus Colocynthis* extract was determined by Folin-Ciocalteu spectrophotometric method according to Bhagyashri *et al.,* (2013). The 0.1 mL of Folin-Ciocalteu reagent was added to 2 mL of the *Citrullus colocynthis* extract. The mixture was allowed to stand for 15 min. Then, 3 mL of saturated sodium carbonate 2% (Na2CO3) was added. The mixture was allowed to stand for 30 min at room temperature and the total phenolic content was determined spectrophotometrically (Labo America, USA) at 760 nm. Gallic acid was used as a standard. Total phenol values are expressedin terms of mg of Gallic acid equivalent per gram of the Sausage sample using the linear regression equation obtained from the standard Gallic acid calibration curve; y = 28.291x + 0.4643. All samples were analyzed in triplicates (Hamad *et al.,* 2018).

6-Total flavonoids content

The total flavonoids content of each aqueous extracts (CCE1, CCE2 and CC3) of C. colocynthis fruits were determined by a colorimetric method. Each sample (0.5 mL) containing 1 mg/mL, was mixed with 2 mL of distilled water and 0.15 mL of NaNO2 solution (15%). After 6 min, 0.15 ml of AlCl3 (10%) and 2 mL of NaOH (4%) solutions were added. Immediately, water was added to bring the final volume to 5 mL and tubes were thoroughly mixed and incubated for 15 min at room temperature. The absorbance was measured at 510 nm. Results were expressed as catechin equivalent per gram of dry extract (mg Cat eq/g) (Benariba *et al.,* 2013; Belkacem *et al.,* 2014).

7- Preparations of beef luncheon as food supplement application

Luncheon rolls meat preparation: The luncheon roll meat batter was prepared according to the following formula: 75% minced beef meat, 10% corn oil, 2.5% salt, 4% gelatin, 1% spices, 1% ground garlic, 2% onion, 4% wheat flour, 0.02% proceed chees isolate and 20% ice. The procedure used in preparation of luncheon roll was carried out according to the protocol described by Ferial *et al.* (2011) with some modifications. Processing of the batter involved blending the frozen minced meat and oil with the other ingredients. The prepared luncheon meat batter was divided into 3 parts 250 gm each with adding 300ppm from CCE1 and CCE2 and one sample without CCE addition. The samples were numeric as following no.1 for CCE1 and 2 For CCE2 sample which was high safety samples in results cytotoxicity determination and 3 for control sample without any additives. Each was mixed well to be homogenous and stuffed into round bottom tubes 5-cm diameter and 15-cm long, fibrous casings (about 250g each) and sealed Luncheon roll meat samples(1-4) were cooked in a air oven set at 100ºC, then held under refrigeration (~1-4ºC) for 10 h. After processing, the luncheon roll meat samples were stored at 4ºC. The sensory evaluation analyses were performed on luncheon samples at different additives.

8-Sensory evaluation:

The sensorial criteria (color .taste, odder, texture and Over all acceptance) of the three (2 high safety samples CCE1, CCE2 and control) luncheon samples under investigation were evaluated by 10 untrained panelists. Luncheon samples were cut into 2mm thick slices and served in numerically-coded glass petri dishes. Each panelist received four coded samples (one from each tested samples) then independently evaluated the luncheon meat for texture, odder, color and taste using a 5-point hedonic scale (0-3 = extremely poor, 4-5 = poor, 6-7= acceptable, 8-9= good, 10= excellent), according to the described method by Lavrova and Krilov, (1975) and Martinez, *et al*. (2004).

Statistical analysis:

The methods of conventional statistical were used to calculate means and standard deviations. All the measurements were replicated 10 times and the data are presented as mean ± SD. The acceptance of panelists data were subjected to analysis SPSS (1997) Significance was defined (0.05> p) (Martinez, *et al.*, 2004).

Results and Discussion

1. Cytotoxicity effect of *C. colocynthis* fruit extract using MTT assay

The data in table 1 showed that the EC100 (ug/ml) of CCE influence on proliferation of wi-38 & vero normal cell line and illustrate effect of EC100 of CCE 1, CCE2 and CCE3 on proliferation of normal wi38 & Vero cell line. Results reveal that CCE1 & 2 exhibit highest EC100 value in both Wi38 & vero normal cell line compared to CCE which give the lowest value in both cell line. It is well known that the highest EC100 value indicates the highest safety of the tested extracts on the proliferation of normal cells. And so, CCE1 & 2 exhibit the safety on the viability of wi38 & Vero cells, with EC100 more 310 and more 450 ug/ml respectively amongst the other tested extract CCE .These results of safety extracts indicate capability to use these two extracts in applied product of food technology as safe ingredient component these results agreed with Jayaraman and Christina (2013) whose reported, no toxic effect was reported at doses up to 7-10 times of effective dose of CCE.

Table 1: The EC_{100} (µg/ml) of *C. colocynthis* fruit extract on the proliferation of normal cells

Samples	Wi-38	Vero
F1	220.83±7.93^{cd}	254.28±2.63^{d}
F2	237.25±1.42^{c}	251.22±2.89^{d}
F3	71.889±7.36^{f}	130.83±1.72^{f}
F4	198.71±0.88^{d}	143.25±2.37^{f}
CCE 1	**313.43±3.32^{a}**	**451.48±6.16^{b}**
CCE 2	**310.36±3.42 ^{a}**	**515.84±3.20^{a}**
CCE 3	259.38±4.93^{bc}	297.10±4.13^{c}
CCE 4	115.30±4.15^{e}	188.05±2.72^{e}

All values are expressed as mean ±standard error of mean (SEM). Different letters indicate significantly different at $p<0.05$.

2. Free radical antioxidant activity by DPPH of CCE

Based on the lowest IC_{50} value of DPPH scavenging activity have the highest antioxidant activity, CCE 1 and CCE 2 exhibited the strongest potential for radical scavenging at < 460 µg/ml comparing to other tested extracts (Table 2). In the present study, the DPPH scavenging activity of the other samples increased in the following order: CCE 4 > CCE 3 > F3 > F4 > F1 > F2. So, F2 had the lowest antioxidant activity amongst all extracts .because the CCE 1, 2 that fist extractions which have more phenolic flavonoid components. This results was in harmony with Sunil (2008).

Table 2:IC_{50} values (µg/ml) for DPPH scavenging activity of *C. colocynthis* fruit extract

Sample	IC_{50}
F1	877.118±3.162^{e}
F2	1527.114±6.11^{f}
F3	813.03±6.73^{d}
F4	863.960±1.35^{e}
CCE 1	438.890±5.6^{a}
CCE 2	458.601±4.919^{a}
CCE 3	715.939±4.151^{c}
CCE 4	630.741±2.461^{b}

3. Antibacterial activity

Data in Table (3) shows antibacterial activity of three CCE against four pathogenic bacteria. Inhibition zones related to CCE show that all extracts have antibacterial activity against studied pathogenic bacteria. On the other hand CCE was more potential against pathogenic bacteria compared to other two extracts CCE1, 2, 3. . Where inhibition zones are 20, 17, 12, 9 compared to 15, 13, 7, 6 and 10, 6, 3, 4 mm against *Bacillus cereus, Staphylococcus aureus*, *E. coli,* & *Clostridium p.* respectively. In addition, *Bacillus cereus* was more affected by CCE compared to other pathogenic bacteria, meanwhile Staph was moderate affected followed by *E. coli* and the lowest affected recorded to Close. These results reveal that CME have different antibacterial activity against pathogenic bacteria depending on pathogenic type and extraction protocol as shown. Moreover, results recommended to

apply CCE as natural antibacterial extract to inhibit most pathogenic bacteria especially bacillus which record highest zone inhibition among all four pathogenic bacteria. In this connection this inhibition effect happened by CCE could be attributed to natural phytochemical constituents found in these extracts that stop or delay bacterial growth. These results are in accordance with Gurudeeban *et al.* (2011) and Gamal *et al.* 2017) who found that ethanolic extract of *Citrullus colocynthus* gives broad spectrum antimicrobial activity against 16 clinical microorganisms included *E. coli* & *Staphylococcus aureus.*

Table 3: Antibacterial activity of aqueous extracts of *Citrullus colocynthis* (100 mg/ml)

Pathogenic strain	Inhibition zone diameter (mm)		
	CCE 1	CCE 2	CCE 3
***Bacillus cereus* EMCC1006**	20	15	10
***Staphylococcus aureus* EMCC1351**	17	13	6
***Escherichia coli* ATCC25922**	12	7	3
***Clostridium perfringens* EMCC1574**	9	6	4

4-Inhibition effect of *Citrullus colocynthis* on pathogenic bacteria.

The results in Table 4 and fig.1 showed that the maximum inhibition zone was of *Bacillus curuse*, *Staphylococcus aureus*, *Escherichia coli,* clostridium per fringe, respectively. And maximum inhibition effect of *Citrullus colocynthis* samples CCE 1, CCE 2, and CCE 3 extract) Respectively .

Table 4: Inhibition zone diameters and MICs of *Citrullus colocynthis* aqueous extract (CCE1) against bacterial strains

Pathogenic strain	Inhibition zone diameter (mm)							
	100*	75*	50*	25*	12.5*	6.2*	3.1*	MIC
***Bacillus cereus* EMCC1006**	20	16	11	6	3	1	ND	6.2
***Staphylococcus aureus* EMCC1351**	17	14	10	5	2	ND	ND	12.5
Escherichia coli ATCC25922	12	9	5	3	ND	ND	ND	25
***Clostridium perfringens* EMCC1574**	9	6	4	2	ND	ND	ND	25

MIC; Minimum Inhibition Concentration *Concentrations of extract and MIC are in mg/mL ND; Not detected.

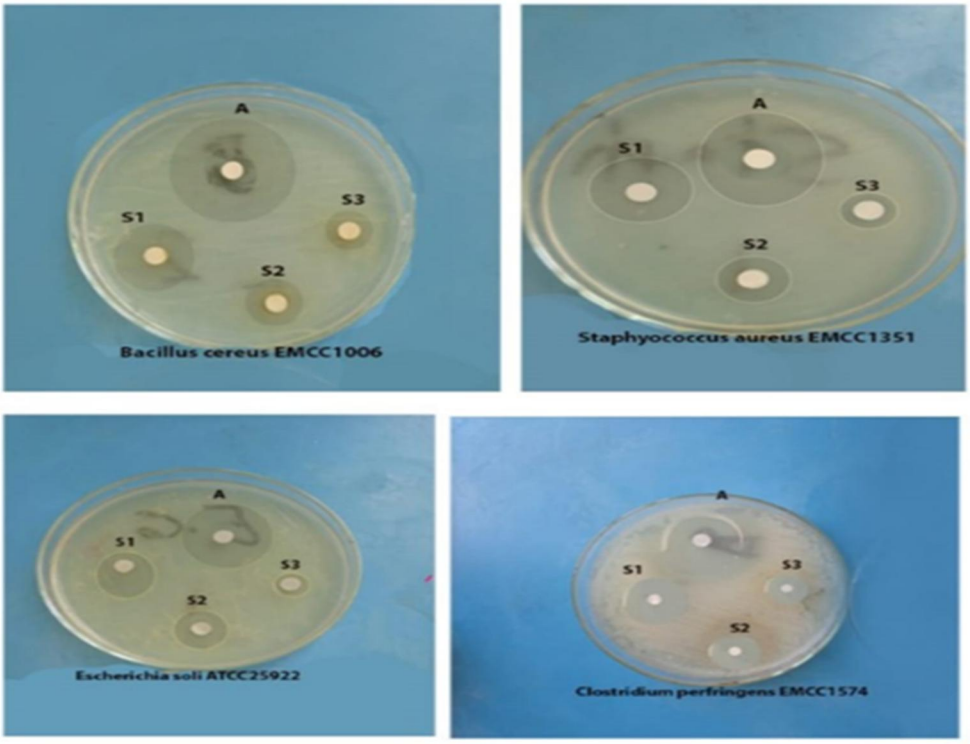

Fig 1: Inhibition zone pictures of *Citrullus colocynthis* aqueous extract CCE1, 2, 3 against bacterial strains in petri dish

Represent MIC of CCE1 which record the best inhibition zone against studied pathogenic bacteria. As shown in Table 5 MIC test was ranged from 100 to 3.1 mg/ml. In addition results reveal that MIC wit 6.2 mg/ml recorded 1 mm iz against *Bacillus cereus*. This result confirm CCE1 have antibacterial activity against all four pathogenic bacteria an *bacillus cereus* was affected by on half of CCE compared to one fold 12.5 in *Staphylococcus aureus* and four fold 25 mg/ml in E.coli and Clostridium p. This wide range of inhibition zones related to different CCE1 concentration permit to use CCE1 to inhibit different pathogenic bacteria depending on kinds of bacteria up to 25 mg/ml. In addition applied this lower concentration as preservative food additive against pathogenic bacteria. This results were agreed with Al-hejjaj *et al.* (2010)

5. Total Phenolic and flavonoids Content

Total Phenolic Content of *Citrullus colocynthis* Extract was found in table(5), it were (1.3748, 0.6981, 0.4790 g) gallic acid equivalent per 100 g aqueous extract to samples CCE 1, CCE 2 and CCE 3 respectively by using the equation y = (28.291x + 0.4643). Results of total phenolic compounds in CCE reveal that phenolic contents were 1.3748, 0.6981, 0.4790 (g/100 ml). These values show that extract No.1 was rich in phenolic compounds compare to extracts 2 & 3. Thus, this high level of phenolic contents in CCE1 will be more strong in protection against oxidative stress either biotic or abiotic stress. Bhagyashri *et al.* (2013).

While the results of the Total flavonoids Content in *Citrullus Colocynthis* Extract found to be 80, 54.33 and 28.33 mg Quercetin equivalent per 100 g aqueous extract to samples CME 1, CCE 2 and CCE 3 respectively by using the equation y = (0.012x - 0.091).

Table 5: Total Phenolic content and Total Flavonoids content of Aqueous extract *Citrullus colocynthis*

Sample	Total Phenolic content (mg/100g)	Total Flavonoids content (mg/100g)
Aqueous extract CCE 1	1.3748	80
Aqueous extract CCE 2	0.6981	54.33
Aqueous extract CCE 3	0.4790	28.33

6. Phytochemical content

Phytochemical constituents in water extract of CM fruit were presented in Table (6). Results reveal that 13 natural compounds were detected in aqueous extract of CM. Preliminary phytochemical screening of the plant extract showed the presence of terpinoids, flavonoids, glycosides, alkaloids and tannins and phenolic this results agreed with Jayaraman and Christina (2013), but they disagreed in glycosides presence may be to used different aqueous solution for extraction in this study we used water for extraction but the other author used methanol

Table 6: Phytochemical screening of highest safety C. colocynthis extract

Chemical Constituent	CCE1	CCE2
Alkaloid	+	+
Tannins	+++	++
Saponins	+	+
Flavanoid	+	+
Terpenoid	++	+
Glycosides	+	+
Steroid	+	+
Flavonoids	+	+
Redusing sugars	-	-
Carbohydrate	+	+
Protein	-	-
Amino Acid	+	+
Total Phenolic compound	+	+

7. Sensory evaluation of luncheon

Data in Table 7. Showed that acceptance grade of sensory evaluation of luncheon with .The CCE1, 2 were moderate acceptable compare with sample control. Sensory parameter include color,

taste, odder, texture and overall acceptance. . In addition, grades of sensory evaluation for CCE1 & 2 gave the same acceptable for panelists and have not significant difference between them. This result reveal that CCE either extracted by soaking or boiling, seeds can caught more bitter components into the plant cells more than which outcome from the plant cells. Generally, the control sample was near panelists acceptance grade with CCE1 (Ferial *et al.,* 2011; Martinez *et al.,* 2004).

Table 7: Sensory evaluation of luncheon content 300 ppm of CCE1, CCE2 and control

Sample no.	Colour (0-10)	Taste (0-10)	Odder (0-10)	Texture (0-10)	Over all acceptance (0-10)
CCE1	5.4[a]	5.8[a]	6.4[a]	6.6[b]	6.1[b]
CCE2	6.6[b]	6.8[a]	6.7[a]	6[b]	6.2[ab]
Control 4	7.3[a]	7.4[b]	7.4[a]	7.4[b]	7.6[a]

Sample 4= control without any extract, Sample 1= with CCE1 extract, Sample 2=with CCE2 extract, All values are mean (SD). Means within column with different letters are significantly different (P<0.05).

Conclusion

The Data Showed that can make a lot of acceptable food applications with *C. colocynthis* extracts CCE1, 2 have moderate sensory evaluation of samples and have a lot of antioxidants, flavonoids and more phenols. We can benefit from the CCE plant as a food supplement, a medicinal compound, and a food preservative.

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