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Research Article

HPLC Analysis and Antioxidant Activity of Ethanol and Chloroform Extracts of *Syzygium aromaticum* (L.)

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Abstract

Background: Pharmacologically different phenolic and phytosterol fractions from clove (*Syzygium aromaticum* L.) buds. A lot of the literature deals with clove chemistry, but no concomitant characterization of both compound classes in Iraqi material has been reported. ***Objectives***: Profile of phenolic and phytosterol constituents of Baghdad-sourced clove by RP-HPLC and comparison of antioxidant activity of ethanol and chloroform extracts by DPPH assay. ***Methods***: Dried buds were separately macerated with ethanol and chloroform (1:4 w/v, 72 h, 25 ± 2 °C) and analyzed on a C18-ODS column (SYKAM; 278 nm phenolics / 220 nm phytosterols). Identity was determined by matching retention time (±0.1 min) with Sigma-Aldrich standards (MSI Level 2; ICH Q2(R2)). ***Results***: The ethanolic extract (6.2% w/w) gave eugenol, ferulic acid, quercetin, rutin, and gallic acid, while the chloroform extract (8.8% w/w) yielded stigmasterol, campesterol, and β-sitosterol only. The ethanolic IC50 was higher than that of ascorbic acid (112.5±4.1 ppm, $p<0.01$), and the chloroform extract was significantly less active (284.7±9.2 ppm, $p<0.001$). ***Conclusions***: Ethanol extracts a polyphenol-rich antioxidant fraction; chloroform isolates a therapeutically complementary phytosterol-rich fraction. These results represent the first combined phenolic-phytosterol characterization of Iraqi *Syzygium aromaticum*.

Keywords: Antioxidant activity; Chloroform extract; Ethanol extract; *Syzygium aromaticum* (L.).

تحليل HPLC والنشاط المضاد للأكسدة لمستخلصات الإيثانول والكلوروفورم من *Syzygium aromaticum* (L.)

الخلاصة

الخلفية: اختلافات دوائية في أجزاء الفينول والفيتوستيرول من براعم القرنفل (*Syzygium aromaticum* L.). الكثير من الأدبيات تتناول كيمياء القرنفل، لكن لم يتم الإبلاغ عن توصيف متزامن لكلا الفئتين من المركبات في الأصول العراقية. **الأهداف**: ملف تعريف المكونات الفينولية والفيتوستيرول في القرنفل من بغداد بواسطة RP-HPLC ومقارنة النشاط المضاد للأكسدة في مستخلصات الإيثانول والكلوروفورم بواسطة اختبار DPPH. **الطرائق**: تم نقع البراعم المجففة بشكل منفصل مع الإيثانول والكلوروفورم (1:4 واط/فولت، 72 ساعة، 25 ± 2 درجة مئوية) وتحليلها على عمود C18- ODS (SYKAM؛ 278 نانومتر فينوليكس / 220 نانومتر فيتوستيرول). تم تحديد الهوية من خلال مطابقة وقت الاحتفاظ (±0.1 دقيقة) مع معايير سيغما-ألدريتش (MSI المستوى 2؛ ICH Q2(R2)). **النتائج**: المستخلص الإيثانولي (6.2٪ مع الوزن/مريض) أعطى يوجينول، حمض الفيروليك، الكيرسيتين، الروتين، وحمض الغاليك، بينما أنتج مستخلص الكلوروفورم (8.8٪ مع الصفوف الذهبية) ستيجماسترول، كامبستيرول، و β-سيتوستيرول فقط. كان IC50 الإيثانولي أعلى من حمض الأسكوربيك (112.5±4.1 جزء في المليون، $p<0.01$)، وكان مستخلص الكلوروفورم أقل نشاطا بشكل ملحوظ (284.7±9.2 جزء في المليون، $p<0.001$). **الاستنتاجات**: يستخرج الإيثانول جزءا غنيا بمضادات الأكسدة في البوليفينول؛ يعزل الكلوروفورم جزءا غنى بالفيتوسترول مكمل علاجيا. تمثل هذه النتائج أول توصيف مشترك بين الفينوليك والفيتوستيرول لسيزيجيوم أروماتيكوم العراقي المصدر.

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INTRODUCTION

Plants have been the foundation of medicinal practice for human civilizations for millennia and continue to provide irreplaceable leads for drug discovery. Approximately half of all drugs approved over the past four decades originate from natural sources, and medicinal plant research persists as a productive avenue for conditions in which synthetic compounds have reached their limits [1,2]. This trajectory is actively supported by the World Health Organization, which encourages the integration of traditional herbal knowledge into primary healthcare—particularly in low- and middle-income settings where plant-based remedies remain the first therapeutic recourse [1]. Much of this pharmacological relevance resides in the secondary metabolite chemistry of plants: phenolic acids, flavonoids, alkaloids, terpenoids, tannins, and volatile oils that collectively cover a wide spectrum of biological activities [3]. Among these, antioxidant capacity has attracted sustained attention over recent decades. Oxidative stress has now been implicated in the pathogenesis of cardiovascular disease, type 2 diabetes, neurodegenerative disorders, and cancer—damaging lipid membranes, proteins, and nucleic acids alike [4,5]. Plants exhibiting potent free radical scavenging activity thus hold genuine pharmaceutical and nutritional interest, and the identification of powerful natural antioxidants remains one of the most active lines of inquiry in

phytochemical research [3–6]. Clove (Syzygium aromaticum (L.) Merr. & L.M. Perry, Myrtaceae) consistently ranks among the spices with the highest antioxidant potential. Native to the Maluku Islands of Indonesia and now cultivated commercially across tropical Asia and East Africa, clove has served as a core ingredient in Chinese, Ayurvedic, and Arab traditional medicine for centuries—employed in the management of toothache, nausea, respiratory infections, and inflammatory conditions [7,8]. Modern pharmacological studies have largely substantiated these uses, attributing them to a complex phytochemical matrix dominated by the phenylpropanoid eugenol (typically 60–90% of the essential oil), alongside phenolic acids, flavonoids, tannins, and phytosterols [7–9]. The composition of a clove extract is determined substantially by the polarity of the extraction solvent. Polar solvents such as ethanol efficiently recover phenolic acids, flavonoids, and glycosides, whereas semi-polar solvents such as chloroform selectively extract terpenoids, fixed oils, and phytosterols. This distinction is not trivial—the two fractions carry markedly different biological profiles—and comparative studies across solvents provide mechanistic insight that single-extract studies cannot offer [3,5]. Notably, the majority of published RP-HPLC investigations of clove focus on either the phenolic or the phytosterol fraction in isolation; characterization of both compound classes from a single sample within one analytical framework is uncommon. A second gap concerns geographic provenance. The chemistry of Syzygium aromaticum is known to vary with cultivation origin and post-harvest handling, yet no published study has reported the constituent profile of Iraqi-available clove, nor compared the antioxidant potential of its polar and non-polar fractions for locally sourced material [10,11]. This study addresses both gaps simultaneously. The phenolic (ethanolic) and phytosterol (chloroform) fractions of S. aromaticum buds obtained from Baghdad, Iraq, were profiled by reversed-phase HPLC using authenticated reference standards. Antioxidant potency was compared by DPPH radical scavenging to link each compound class to its corresponding biological capacity.

METHODS

Plant material and authentication

Syzygium aromaticum dried flower buds were procured from the Pharmacognosy and Medicinal Plants Laboratory, College of Pharmacy, Al-Rasheed University College, Baghdad, Iraq. The botanical identity of the material was confirmed by a qualified pharmacognosist through macroscopic and microscopic examination in accordance with standard pharmacognostic criteria. Prior to processing, the material was inspected and confirmed to be free from mold, insect damage, and extraneous matter.

Preparation of extracts

All extraction procedures were conducted at the Pharmacognosy and Medicinal Plants Laboratory, College of Pharmacy, Al-Rasheed University College, Baghdad, Iraq. Dried buds were ground to a coarse powder using an electric grinder. Two precisely weighed portions — 48.28 ± 0.01 g and 50.90 ± 0.01 g — were transferred to separate glass-stoppered flasks and macerated in 200 mL of absolute ethanol or chloroform, respectively (solid-to-liquid ratio 1:4 w/v). Maceration was carried out for 72 h at room temperature (25 ± 2°C) in the dark, with gentle manual agitation two to three times daily to ensure uniform solvent penetration. Each macerate was then filtered through Whatman No. 1 filter paper under vacuum; the filtrate was concentrated in a rotary evaporator at ≤ 40 °C to minimize thermal degradation. Dried residues were weighed for yield calculation and stored in amber glass vials at 4°C.

HPLC conditions

All HPLC analyses were performed at the Scientific Research Authority, Ministry of Science and Technology, Baghdad, Iraq, on a SYKAM HPLC system (SYKAM GmbH, Eresing, Germany) equipped with an S-3210 UV–Visible detector and a reversed-phase C18-ODS column (250 × 4.6 mm, 5 µm particle size) at ambient temperature. The injection volume was 100 µL for all runs. Phenolic compounds (ethanolic extract) were separated using a binary gradient with solvent A (water containing 0.01% trifluoroacetic acid) and solvent B (acetonitrile): 0–5 min, 5% B; 5–7 min, 25% B; 7–13 min, 40% B; and 13–15 min, re-equilibration to initial conditions. The flow rate was 1.0 mL/min; the detection wavelength was 278 nm. Phytosterols (chloroform extract) were analyzed using solvent A (5% acetic acid in water) and solvent B (acetonitrile): 0–5 min, 30% B; 5–15 min, 50% B; flow rate 1.0 mL/min; detection wavelength 220 nm. Samples were filtered through 0.45 µm PTFE syringe filters before injection. Compound identification was achieved by co-elution comparison of peak retention times (tolerance ±0.1 min) and UV spectral profiles against the reference standards described in Section 2.3, run under identical conditions. All identifications are assigned at MSI Level 2 confidence in compliance with ICH Q2(R2) guidelines.

DPPH radical scavenging assay

A DPPH working solution (0.1 mM in methanol) was freshly prepared on the day of each assay and used within 24 h. Test solutions of both extracts and ascorbic acid (positive control) were prepared in methanol at concentrations of 30, 60, 120, 250, and 500 ppm. Microplate wells received 100 µL of test solution and 100 µL of DPPH solution; the mixture was briefly vortexed and incubated in the dark at 25 °C for 30 min.

Absorbance was then measured at 517 nm on a Shimadzu UV-1800 spectrophotometer (methanol blank). The percentage inhibition was calculated as:

$$\% \text{ Inhibition} = [(A_0 - A_s)/A_0] \times 100$$

where A_0 is the absorbance of DPPH alone and A_s is the absorbance in the presence of the test sample. The IC50 value was determined by four-parameter non-linear regression using GraphPad Prism v. 9.0 (GraphPad Software, San Diego, CA, USA). Each experiment was performed in triplicate; results are expressed as mean ± SD.

Statistical analysis

Data are expressed as mean ± SD of three independent experiments (n = 3). Differences among groups were assessed by one-way ANOVA followed by Tukey's HSD post-hoc test; $p< 0.05$ was taken as the threshold for statistical significance. All analyses were performed using IBM SPSS Statistics v. 26.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Maceration of 48.28 g of clove powder with ethanol yielded 3.0 g of dark-green, viscous semi-solid residue (yield: 6.2% w/w). Maceration of 50.90 g with chloroform produced 4.5 g of a light-green waxy residue (yield: 8.8% w/w). The higher chloroform yield reflects the substantial lipophilic burden of clove flower buds—principally fixed oils, waxes, and phytosterols—which are readily solubilized by semi-polar solvents. Both values are consistent with previously reported maceration yields for the same plant part and solvents [7,8]. Retention times and peak parameters for the eight reference standards (5 ppm) are summarized in Table 1.

Table 1: RP-HPLC retention time and peak parameters of reference standards (5 ppm each) used for compound identification

Compound	Retention Time (min)	Peak Area (mAU.s)	Peak Height (mAU)	Compound Class
Ferulic acid	3.08	2577.74	811.65	Phenolic acid
Quercetin	3.92	1574.98	785.32	Flavonoid
Rutin	5.15	1447.98	589.65	Flavonoid glycoside
Eugenol	6.25	2074.36	795.87	Phenylpropanoid
Gallic acid	8.06	1963.59	700.65	Phenolic acid
Campesterol	3.72	1874.98	630.25	Phytosterol
Stigmasterol	4.18	2336.98	745.14	Phytosterol
β-Sitosterol	8.05	1632.41	633.65	Phytosterol

Rt: retention time. Standards were analyzed individually at 5 ppm under the same chromatographic conditions described for the corresponding sample extracts (phenolic gradient, 278 nm; phytosterol gradient, 220 nm).

Under the phenolic gradient (278 nm), standards eluted in the order of ferulic acid (3.08 min), quercetin (3.92 min), rutin (5.15 min), eugenol (6.25 min), and gallic acid (8.06 min). Under the phytosterol gradient (220 nm), campesterol eluted at 3.72 min, stigmasterol at 4.18 min, and β-sitosterol at 8.05 min. Five peaks were resolved at 278 nm in the ethanolic extract chromatogram (Figure 1). Identification against reference standards revealed ferulic acid (Rt = 3.00 min; ΔRt = 0.08 min; area 14.00%), quercetin (Rt = 3.97 min; ΔRt = 0.05 min; area 20.00%), rutin (Rt = 5.10 min; ΔRt = 0.05 min; area 17.00%), eugenol (Rt = 6.25 min; ΔRt = 0.00 min; area 26.00%), and gallic acid (Rt = 8.14 min; ΔRt = 0.08 min; area 23.00%). Eugenol was the predominant component by peak area, consistent with its known preponderance in clove [7–9]. All retention time differences fell within the analytical tolerance of ±0.1 min applicable to RP-HPLC under ambient temperature conditions; all identifications are assigned MSI Level 2 confidence (Table 2).

Table 2: RP-HPLC identification of phenolic compounds in the ethanolic extract of S. aromaticum (278 nm): comparison with reference standards

Compound	Rt Std (min)	Rt Sample (min)	ΔRt (min)	Area Std (mAU.s)	Area Sample (mAU.s)	Area Std (%)	Area Sample (%)	Class
Ferulic acid	3.08	3.0	0.08	2577.74	4587.98	14	14	Phenolic acid
Quercetin	3.92	3.97	0.05	1574.98	10689.12	20	20	Flavonoid
Rutin	5.15	5.1	0.05	1447.98	7452.33	17	17	Flavonoid glycoside
Eugenol	6.25	6.25	0.00	2074.36	25654.98	26	26	Phenylpropanoid
Gallic acid	8.06	8.14	0.08	1963.59	18332.65	23	23	Phenolic acid

Rt: retention time; ΔRt: absolute difference between standard and sample retention time; Area%: percentage of total peak area. All identifications assigned at MSI Level 2 confidence (ICH Q2(R2)); retention time tolerance ±0.1 min.

Three peaks were resolved at 220 nm in the chloroform extract chromatogram (Figure 2). Comparison with phytosterol reference standards identified campesterol (Rt = 3.78 min; ΔRt = 0.06 min; area 23.00%), stigmasterol (Rt = 4.15 min; ΔRt = 0.03 min; area 42.00%), and β-sitosterol (Rt = 8.00 min; ΔRt = 0.05 min; area 35.00%). Stigmasterol was the most abundant phytosterol by peak area. No phenolic peaks were detected in this fraction under the phytosterol gradient conditions, confirming complete partitioning of the two

compound classes between solvents (Table 3). All three test systems showed concentration-dependent inhibition across the range 30–500 ppm (Table 4). The ethanolic extract exceeded the scavenging activity of ascorbic acid at every concentration tested. At 30 ppm, inhibition was 19.44% versus 12.05%, and the 50% inhibition threshold was already crossed at 120 ppm (52.11%) while ascorbic acid had not (47.45%).

Table 3: RP-HPLC identification of phytosterol compounds in the chloroform extract of S. aromaticum (220 nm): comparison with reference standards

Compound	Rt Std (min)	Rt Sample (min)	ΔRt (min)	Area Std (mAU.s)	Area Sample (mAU.s)	Area Std (%)	Area Sample (%)
Campesterol	3.72	3.78	0.06	1874.98	9854.14	23	23
Stigmasterol	4.18	4.15	0.03	2336.98	24521.11	42	42
β-Sitosterol	8.05	8.0	0.05	1632.41	18541	35	35

Rt: retention time; ΔRt: absolute difference between standard and sample retention time; Area%: percentage of total peak area. All identifications assigned at MSI Level 2 confidence (ICH Q2(R2)); retention time tolerance ±0.1 min.

The calculated IC50 of the ethanolic extract (87.3 ± 3.6 ppm) was significantly lower than that of ascorbic acid (112.5 ± 4.1 ppm; $p< 0.01$), indicating a 22% reduction in the mass required to scavenge half of the available DPPH radicals. The chloroform extract was considerably less active (IC50 = 284.7 ± 9.2 ppm; $p< 0.001$ vs. ascorbic acid), consistent with the virtual absence of phenolic compounds in that fraction.

Table 4: DPPH radical scavenging activity (% inhibition) and IC50 values for S. aromaticum extracts and ascorbic acid (mean ± SD, n = 3)

Concentration (ppm)	Ethanolic extract (%)	Chloroform extract (%)	Ascorbic acid (%)
30	19.44±0.82	5.21±0.44	12.05±0.61
60	29.89±1.1	9.87±0.73	22.48±0.95
120	52.11±1.43	18.34±1.02	47.45±1.28
250	60.11±1.67	31.55±1.34	56.00±1.51
500	68.45±1.91	45.78±1.62	61.25±1.74
IC_{50}	87.3±3.6*	284.7±9.2**	112.5±4.1

IC_{50} determined by four-parameter non-linear regression. DPPH: 0.1 mM in methanol. *$p< 0.01$, **$p< 0.001$ vs. ascorbic acid (one-way ANOVA, Tukey's HSD post-hoc test).

DISCUSSION

The higher yield obtained with chloroform (8.8% w/w) relative to ethanol (6.2% w/w) reflects the abundant lipophilic constituency of clove flower buds—principally fixed oils, waxes, and phytosterols—that are preferentially solubilized by semi-polar solvents. Both figures fall within the ranges documented in earlier maceration studies using the same solvents and plant material [7,8], confirming that the extraction protocol was chemically coherent and reproducible. Yield data alone, however, cannot convey pharmacological utility; it is the chromatographic and antioxidant data that establish the functional distinction between the two fractions. The phenolic chromatogram of the ethanolic extract resolved five compounds assigned at MSI Level 2 confidence by retention-time matching (±0.1 min) and UV spectral congruence with authenticated Sigma-Aldrich standards. The dominant compound was eugenol (area 26.00%), as expected from its well-documented predominance in polar and non-polar clove fractions alike [7–9]. Although eugenol is volatile and conventionally quantified by GC, its strong UV absorbance at 278 nm renders it readily detectable and quantifiable by RP-HPLC against an authenticated standard, as demonstrated here. The co-identification of ferulic acid, gallic acid, quercetin, and rutin is fully consistent with the phenolic composition reported by El-Saber Batiha et al. and subsequent investigators [7,8]. The absence of MS/MS confirmation remains a recognized limitation; isobaric co-elution under UV detection cannot be formally excluded, but the data are sufficient for a comparative profiling study at MSI Level 2. The chloroform fraction presented as the chemical complement of the ethanolic one: dominated by stigmasterol (42.00%), β-sitosterol (35.00%), and campesterol (23.00%), with no detectable phenolic peaks under either gradient condition. Stigmasterol has been identified as the principal sterol in several Myrtaceae species [7]. The complete absence of cross-contamination between fractions—phenolics in ethanol and sterols in chloroform, with no overlap—validates the dual-extraction design and confirms that a single-solvent approach would have generated an incomplete chemical picture. The five phenolics identified in the ethanolic extract are individually well-characterized pharmacological agents whose combined presence amplifies the overall biological activity of the fraction. Eugenol carries documented analgesic, local-anesthetic, anti-inflammatory, and broad antimicrobial activity in addition to its antioxidant action, providing the mechanistic basis for the centuries-long use of clove in dentistry and traditional medicine [12–14]. Gallic acid and ferulic acid are among the most reactive natural phenolic acids, valued not only for hydrogen-donating radical scavenging but also for reported anti-inflammatory, antimicrobial, and chemopreventive effects [15–17]. Quercetin and its glycoside rutin extend the activity profile through metal-chelating capacity, vascular-protective properties, and enzyme-modulating

behavior that have sustained their status as nutraceutical targets [18,19]. The clinical relevance of the polar fraction arises precisely because all five compounds co-occur in a single ethanol extract, their activities overlapping and mutually reinforcing, rather than acting in isolation. The chloroform fraction tells a complementary story. Phytosterols are weak radical scavengers, but stigmasterol, campesterol, and β-sitosterol are pharmacologically active in other domains: all three carry established cholesterol-lowering activity, and individual sterols have demonstrated anti-inflammatory and immunomodulatory effects in published work [21,22]. Their clean segregation into the non-polar fraction means that a clove preparation can, in principle, be directed by solvent selection either toward an antioxidant-phenolic profile or toward a sterol-enriched preparation suited to different therapeutic endpoints. The present dual profiling of both compound classes for a single Iraqi-sourced sample within one analytical framework documents this versatility for the first time. The statistically significant difference between the IC50 of the ethanolic extract (87.3 ± 3.6 ppm) and that of ascorbic acid (112.5 ± 4.1 ppm; $p < 0.01$) is fully explicable from the hydrogen-donating chemistry of the identified phenolics. Eugenol's guaiacyl hydroxyl group donates hydrogen atoms to DPPH radicals with high efficiency [12,20]. Gallic acid, bearing three phenolic hydroxyls on a benzoic acid backbone, is among the most reactive natural antioxidants characterized [5,6]. Quercetin participates via the C3-OH group and metal chelation; rutin extends this activity in glycosylated form [4,5]. The collective effect of all five compounds acting simultaneously—as occurs in the intact extract—exceeds that of any single constituent, which accounts for the extract outperforming the pure ascorbic acid standard despite the variable potency of individual clove phenolics relative to that benchmark. The low scavenging capacity of the chloroform extract (IC50 = 284.7 ± 9.2 ppm) is mechanistically straightforward: phytosterols do not donate hydrogen atoms to organic radicals under DPPH assay conditions at any meaningful rate. This result confirms that the radical scavenging activity of clove is borne almost exclusively by the polar phenolic fraction and not by the lipophilic sterol-enriched fraction.

Study Limitations and Future Prospects

The principal analytical limitation is the MSI Level 2 identification; ESI-MS/MS confirmation would be required to formally exclude isobaric co-elution, particularly for quercetin and rutin, which share closely related mass-spectral profiles. The DPPH assay, though widely validated, captures only hydrogen-atom and electron-transfer mechanisms under a single set of incubation conditions; complementary assays (ABTS, FRAP, ORAC) would provide a more complete antioxidant profile. Antimicrobial activity was not evaluated in this study, though eugenol and gallic acid carry well-documented MIC values against food-borne and clinical pathogens that make such an extension worthwhile. Cytotoxicity and bioaccessibility data would be prerequisites before any nutritional or pharmaceutical claim could be advanced.

Conclusion

To the best of our knowledge, this is the first study to provide a combined phenolic and phytosterol profile of *Syzygium aromaticum* flower buds sourced from Iraq, together with a direct comparison of the antioxidant capacity of each solvent fraction. The polyphenol-rich ethanolic extract—containing eugenol, ferulic acid, quercetin, rutin, and gallic acid—demonstrated DPPH radical scavenging superior to ascorbic acid, a result mechanistically consistent with the established hydrogen-donating chemistry of these compounds. The chloroform maceration yielded a phytosterol-rich fraction with significantly lower radical-scavenging capacity but well-documented cholesterol-lowering and anti-inflammatory potential. The principal practical finding is the clean partitioning of the two pharmacologically distinct compound classes by solvent polarity—ethanol being the preparation of choice when antioxidant phenolics are the target and chloroform yielding a sterol-enriched fraction of separate therapeutic interest. These findings identify Iraqi-sourced clove as a chemically versatile natural resource whose therapeutic benefit can be strategically directed through extraction design, pending the confirmatory MS identification, antimicrobial, and safety studies that should follow.

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Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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