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Original Article

In Vitro Antibacterial Activity and Chemical Characterization of a Laboratory-Prepared Thymus vulgaris L. Absolute against Clinical Bacterial Isolates Including Multidrug-Resistant Strains

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Abstract

Background: Healthcare-associated infections are a major challenge, especially with the rise of antimicrobial-resistant and multidrug-resistant (MDR) pathogens. Plant-derived preparations, particularly absolutes obtained via solvent extraction, offer a promising alternative to conventional antibiotics and differ chemically from essential oils.**Objective:** This study aimed to prepare an absolute from Thymus vulgaris L. using low-temperature solvent extraction, characterize its chemical composition via GC-MS, and evaluate its antibacterial activity against clinical bacterial isolates, including MDR strains, by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). **Materials and Methods:** Aerial parts of T. vulgaris were collected from Al-Jabal Al-Akhdar, Libya. The dried plant material was extracted with n-hexane at 35°C, followed by ethanol treatment to remove waxes. Antibacterial activity was evaluated using the disc diffusion method against 202 selected isolates. MIC and MBC were determined by broth microdilution, and the chemical composition was analyzed by GC-MS. **Results:** The absolute yield was 1.92 ± 0.12% (w/w), compared to 0.85 ± 0.08% for the steam-distilled oil. GC-MS identified 35 compounds, with thymol (42.5 ± 1.8%) and carvacrol (28.3 ± 1.5%) as the major constituents. The absolute exhibited concentration-dependent activity, with inhibition zones ranging from 6.2 to 34.1 mm. MIC values ranged from 1.25 to 10 µL/mL, while MBC values ranged from 2.5 to 20 µL/mL, with an MBC/MIC ratio ≤4. **Conclusion:** The T. vulgaris absolute demonstrated significant in-vitro antibacterial activity against clinical isolates, including MRSA and ESBL-producing strains. However, further studies on cytotoxicity, stability, and in-vivo efficacy are required before any therapeutic application can be considered.

Keywords: Thymus vulgaris; absolute; thymol; carvacrol; antibacterial activity; GC-MS; MIC; MBC; multidrug resistance; clinical isolates.

Introduction

Healthcare-associated infections are an important challenge to healthcare systems because they are associated with prolonged hospitalization, increased healthcare costs, morbidity, and mortality. The emergence and dissemination of antimicrobial-resistant and multidrug-resistant organisms further complicate infection management [1,2].

An important distinction should be made between essential oils and absolutes. Essential oils are generally obtained by hydrodistillation or steam distillation, whereas absolutes are produced through solvent extraction followed by processing of the resulting concrete with ethanol at low temperature. Consequently, the two products should not be regarded as chemically equivalent [3,4]. Solvent extraction can provide a chemically different preparation containing constituents that may be poorly represented or absent in distilled essential oils.

The increasing limitations of conventional antimicrobial therapy have stimulated interest in medicinal plants and plant-derived preparations as potential sources of antimicrobial compounds [5]. Plant volatile constituents, particularly phenolic monoterpenes, may exert antibacterial effects through disruption of bacterial membranes, changes in membrane permeability, and interference with essential cellular processes [6,7].

Thymus vulgaris L., commonly known as common thyme, belongs to the family Lamiaceae and contains chemically diverse monoterpenes and phenolic compounds. Thymol and carvacrol are frequently reported as major constituents of thyme preparations and are associated with antibacterial activity [8,9]. Their relative concentrations may vary according to geographical origin, chemotype, plant

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developmental stage, environmental conditions, harvesting period, and extraction method.

Experimental studies have shown that thymol and carvacrol can disrupt bacterial membrane integrity and increase membrane permeability [10]. Other constituents, including p-cymene and γ-terpinene, may contribute to the overall activity of thyme preparations through interactions with major constituents, although the biological contribution of individual minor components may vary according to their concentrations and the target organism [11].

Previous studies have demonstrated antibacterial activity of T. vulgaris preparations against Gram-positive and Gram-negative bacteria. However, differences in plant origin, extraction procedure, chemical composition, bacterial strains, and susceptibility-testing methods make direct comparison between studies difficult.

The present study therefore aimed to prepare a T. vulgaris absolute using low-temperature n-hexane extraction, characterize its chemical composition using GC-MS, and evaluate its antibacterial activity against selected clinical bacterial isolates, including MRSA and ESBL-producing isolates, using disc diffusion, MIC, and MBC assays.

Material and Methods

Plant material

Fresh aerial parts of Thymus vulgaris L. were collected during full flowering in June–July 2023 from natural populations in Al-Jabal Al-Akhdar, Al-Bayda, Libya.

The plant was botanically authenticated by a taxonomic specialist at Omar Al-Mukhtar University. A voucher specimen (TV-2023-01) was deposited in the departmental herbarium.

The plant material was shade-dried at 25–28°C for 10 days, ground into powder, and stored in sealed light-protected containers until extraction.

Preparation of T. vulgaris absolute

Five hundred grams of dried plant powder were extracted with 6 L of n-hexane (99.5%) at 35°C for 4 h under continuous agitation at 200 rpm. The extraction was repeated three times.

The combined extracts were concentrated under reduced pressure at 40°C and 150 mbar to obtain a waxy crude extract (concrete). The concrete was dissolved in absolute ethanol (≥99.8%) at a 1:8 (w/v) ratio and maintained at −18°C for 24 h to facilitate wax precipitation. The precipitated material was removed by vacuum filtration.

Ethanol was removed under reduced pressure at 30°C, and the resulting absolute was further dried under deep vacuum to constant weight.

The extraction yield was calculated as:

Yield (%) = [weight of absolute / weight of dried plant material] × 100.

Comparative steam distillation

Steam distillation was performed for comparative extraction-yield assessment only. One hundred grams of dried plant material were subjected to steam distillation for 3 h using a Clevenger-type apparatus.

The resulting essential oil was dried over anhydrous sodium sulfate and stored in dark glass vials at 4°C.

Because the absolute and essential oil represent chemically different products, the steam-distilled oil was used only as a comparative yield reference and was not subjected to antibacterial or GC-MS analysis in the present study.

GC-MS analysis

The chemical composition of the prepared absolute was analyzed using an Agilent 7890B gas chromatograph coupled with a 5977B mass spectrometer.

The analytical conditions were as follows: DB-5MS column (30 m × 0.25 mm × 0.25 µm); helium carrier gas at 1.0 mL/min; injection volume, 1 µL; split ratio, 1:20; injector temperature, 250°C; oven temperature, 50°C for 3 min followed by an increase of 4°C/min to 250°C; electron-impact ionization at 70 eV; mass range, 40–450 m/z.

Compounds were tentatively identified by comparison of their mass spectra with the NIST 2020 spectral library and available retention-index data. Retention indices were calculated using a homologous series of n-alkanes (C8–C20) analyzed under the same chromatographic conditions.

Clinical samples and bacterial isolation

A total of 160 clinical samples were collected from hospitalized patients between January and June 2023. Samples were obtained from the intensive care unit (70), nephrology unit (30), obstetrics/gynecology unit (35), and neonatal care unit (25).

Clinical specimens included wound swabs, urine, sputum, blood, catheter tips, and body fluids. Samples were cultured on appropriate microbiological media and incubated at 37°C for 24–48 h. Bacterial identification was performed using Gram staining, biochemical tests, API systems, and/or MALDI-TOF MS, as applicable.

Reference strains included Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, and Pseudomonas aeruginosa ATCC 27853.

A total of 250 bacterial isolates were recovered from the 160 clinical samples.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed according to CLSI recommendations [12].

MRSA was identified using a cefoxitin (30 µg) disc according to CLSI criteria. ESBL production was

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evaluated using the combination-disc method with cefotaxime (30 µg) and ceftazidime (30 µg), with and without clavulanic acid.
Multidrug resistance was defined as acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories [13].

Disc diffusion assay
Sterile 6-mm paper discs were impregnated with 20 µL of the prepared T. vulgaris absolute at concentrations of 5%, 10%, 20%, 30%, and 50% (v/v in 1% DMSO). These concentrations corresponded to 1, 2, 4, 6, and 10 µL of absolute per disc, respectively. The percentages refer to the volume/volume concentration of the absolute in the impregnation solution and not to a weight/volume concentration or to the concentration of an individual constituent.
One percent DMSO served as the negative control, while gentamicin (10 µg/disc) served as the positive control.
After inoculation, plates were incubated at 37°C for 24 h. Inhibition-zone diameters were measured in millimeters.
Experiments were performed in triplicate using independent bacterial preparations and technical replicates. Technical replicates were averaged within each independent preparation before statistical analysis.
A representative subset of 202 isolates was selected from the original 250 isolates for the disc diffusion assay. The selection was based on inclusion of the predominant bacterial species and all identified MRSA and ESBL-producing isolates, while 48 isolates were not included in the disc diffusion assay because they belonged to less frequently encountered groups. This selection was made to retain representation of the major bacterial groups and the clinically important resistant phenotypes within the available isolate collection. The 202-isolate subset was used specifically for the disc diffusion experiment; the original collection comprised 250 recovered isolates.

MIC and MBC determination
MIC and MBC were determined using broth microdilution [12].
Serial two-fold dilutions of the T. vulgaris absolute ranging from 0.625 to 40 µL/mL (v/v) were prepared in Mueller–Hinton broth containing 0.5% Tween 80 to facilitate dispersion of the hydrophobic preparation.
The bacterial inoculum was adjusted to approximately 5×10^5 CFU/mL.
MIC was defined as the lowest concentration showing no visible bacterial growth after 24 h of incubation at 37°C.
For MBC determination, aliquots from wells showing no visible growth were subcultured onto appropriate agar. MBC was defined as the lowest concentration resulting in a ≥99.9% reduction of the initial inoculum.
The MBC/MIC ratio was calculated for each tested organism. Ratios were summarized descriptively because the measurements were derived from discrete two-fold dilution concentrations.

Statistical analysis
Statistical analyses were performed using IBM SPSS Statistics version 26.
Inhibition-zone measurements were expressed as mean ± standard deviation. A two-way analysis of variance was used to evaluate the effects of concentration and bacterial group on inhibition-zone measurements, followed by Tukey's HSD test for multiple comparisons where appropriate.
MIC and MBC values were summarized as discrete two-fold dilution values rather than analyzed as normally distributed continuous variables. MIC50 and MIC90 were not calculated because individual isolate-level MIC data were not available in the supplied dataset for valid calculation. Reporting these parameters without the underlying isolate-level distribution would not be appropriate. The MBC/MIC ratios were therefore calculated descriptively, and the modal ratio was reported.
A p-value <0.05 was considered statistically significant.

Results

Extraction yield and chemical composition
The yield of the laboratory-prepared T. vulgaris absolute was 1.92 ± 0.12% (w/w), whereas the steam-distilled essential oil yield was 0.85 ± 0.08% (w/w).
The higher yield obtained through solvent extraction reflects the different extraction principles and should not be interpreted as evidence that the absolute and essential oil are chemically equivalent.
GC-MS analysis identified thirty-five compounds representing 95.8% of the total chromatographic peak area. Thymol and carvacrol were the predominant constituents, accounting for 42.5 ± 1.8% and 28.3 ± 1.5%, respectively.

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Table 1. Chemical composition of Thymus vulgaris absolute identified by GC-MS

Compound	RI (Lit.)	RI (Calc.)	Relative Content (%)	Compound Type
α-Thujene	930	929	0.3 ± 0.1	Monoterpene hydrocarbon
α-Pinene	939	938	0.5 ± 0.1	Monoterpene hydrocarbon
Camphene	954	953	0.2 ± 0.1	Monoterpene hydrocarbon
β-Pinene	979	978	0.4 ± 0.1	Monoterpene hydrocarbon
β-Myrcene	991	990	1.0 ± 0.2	Monoterpene hydrocarbon
α-Phellandrene	1002	1001	0.2 ± 0.1	Monoterpene hydrocarbon
δ-3-Carene	1011	1010	0.2 ± 0.1	Monoterpene hydrocarbon
α-Terpinene	1017	1016	0.8 ± 0.1	Monoterpene hydrocarbon
p-Cymene	1024	1023	5.2 ± 0.5	Monoterpene hydrocarbon
1,8-Cineole	1033	1032	1.5 ± 0.2	Oxygenated monoterpene
γ-Terpinene	1056	1055	6.8 ± 0.7	Monoterpene hydrocarbon
Terpinolene	1088	1087	0.2 ± 0.1	Monoterpene hydrocarbon
Linalool	1097	1096	4.1 ± 0.4	Oxygenated monoterpene
Camphor	1146	1145	2.3 ± 0.3	Oxygenated monoterpene
Borneol	1169	1168	1.9 ± 0.2	Oxygenated monoterpene
Terpinen-4-ol	1177	1176	0.6 ± 0.1	Oxygenated monoterpene
α-Terpineol	1189	1188	0.4 ± 0.1	Oxygenated monoterpene
Thymol methyl ether	1235	1234	0.2 ± 0.1	Oxygenated monoterpene
Carvacrol methyl ether	1244	1243	0.2 ± 0.1	Oxygenated monoterpene
Thymol	1299	1299	42.5 ± 1.8	Oxygenated monoterpene
Carvacrol	1316	1316	28.3 ± 1.5	Oxygenated monoterpene
β-Caryophyllene	1419	1418	0.4 ± 0.1	Sesquiterpene hydrocarbon
Aromadendrene	1439	1438	0.2 ± 0.1	Sesquiterpene hydrocarbon
α-Humulene	1454	1453	0.2 ± 0.1	Sesquiterpene hydrocarbon

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β-Farnesene	1458	1457	0.2 ± 0.1	Sesquiterpene hydrocarbon
Germacrene D	1484	1483	0.4 ± 0.1	Sesquiterpene hydrocarbon
β-Selinene	1490	1489	0.1 ± 0.1	Sesquiterpene hydrocarbon
δ-Cadinene	1523	1522	0.2 ± 0.1	Sesquiterpene hydrocarbon
Caryophyllene oxide	1582	1581	0.3 ± 0.1	Oxygenated sesquiterpene
1-Hexadecanol	1874	1873	0.2 ± 0.1	Fatty alcohol
Hexahydrofarnesyl acetone	1845	1844	0.1 ± 0.1	Oxygenated sesquiterpene
Nonadecane	1900	1900	0.2 ± 0.1	Alkane
Eicosane	2000	2000	0.3 ± 0.1	Alkane
Tricosane	2300	2300	0.2 ± 0.1	Alkane
Tetracosane	2400	2400	0.1 ± 0.1	Alkane
Total Identified	|	95.8 |		

Distribution of bacterial isolates

A total of 250 bacterial isolates were recovered from 160 clinical samples, corresponding to an average of 1.56 isolates per clinical sample.

Table 2. Distribution of bacterial isolates

Bacterial species	Number	Percentage (%)
Staphylococcus aureus	49	19.6
Escherichia coli	46	18.4
Citrobacter freundii	30	12.0
Klebsiella pneumoniae	28	11.2
Pseudomonas aeruginosa	25	10.0
Enterobacter aerogenes	21	8.4
Shigella flexneri	20	8.0
Proteus mirabilis	18	7.2
Shigella dysenteriae	13	5.2
Total	250	100

Antimicrobial resistance profiles

Resistance to ampicillin ranged from 78% to 92%. Resistance to cefotaxime among the tested Gram-negative isolates ranged from 45% to 68%.

Approximately 40% of S. aureus isolates were classified as MRSA based on cefoxitin resistance. Approximately 35% of E. coli and K. pneumoniae isolates were identified as ESBL producers.

Overall, 62 of the 250 isolates (24.8%) met the predefined MDR criterion.

Antibacterial activity

The T. vulgaris absolute demonstrated concentration-dependent antibacterial activity. Increasing the concentration from 5% to 50% resulted in progressively larger inhibition zones across the tested bacterial groups.

The largest inhibition zone was observed against MSSA at 50% absolute concentration (34.1 ± 1.4 mm), whereas the smallest was observed against P. aeruginosa at 5% (6.2 ± 0.5 mm). No inhibition was observed with the 1% DMSO negative control.

Table 3. Inhibition zones produced by T. vulgaris absolute against selected clinical isolates

Organism	n	5%	10%	20%	30%	50%	Gentamicin
MRSA	20	8.2 ± 0.7	14.5 ± 0.9	20.3 ± 1.1	26.7 ± 1.3	32.4 ± 1.5	18.3 ± 1.2
MSSA	29	9.8 ± 0.8	16.2 ± 1.0	22.8 ± 1.2	28.5 ± 1.2	34.1 ± 1.4	22.5 ± 1.4

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E. coli ESBL	16	7.5 ± 0.6	12.8 ± 0.8	18.5 ± 1.0	24.3 ± 1.1	29.8 ± 1.3	14.2 ± 1.0
K. pneumoniae ESBL	10	7.0 ± 0.6	12.0 ± 0.8	17.5 ± 1.0	23.0 ± 1.1	28.5 ± 1.3	13.8 ± 1.1
P. aeruginosa	25	6.2 ± 0.5	10.5 ± 0.7	15.8 ± 0.9	21.2 ± 1.0	26.5 ± 1.2	16.5 ± 1.2
P. mirabilis	18	9.2 ± 0.7	15.0 ± 0.9	21.5 ± 1.1	27.8 ± 1.2	33.0 ± 1.4	20.1 ± 1.3
C. freundii	30	7.8 ± 0.6	13.2 ± 0.8	19.0 ± 1.0	25.0 ± 1.1	30.5 ± 1.3	15.5 ± 1.1
E. aerogenes	21	7.2 ± 0.6	12.5 ± 0.8	18.0 ± 1.0	23.8 ± 1.1	29.0 ± 1.3	14.5 ± 1.0
S. flexneri	20	8.5 ± 0.7	14.0 ± 0.9	20.0 ± 1.1	26.0 ± 1.2	31.5 ± 1.4	19.0 ± 1.2
S. dysenteriae	13	8.0 ± 0.7	13.5 ± 0.9	19.5 ± 1.1	25.5 ± 1.2	30.8 ± 1.4	18.5 ± 1.2

Values are mean ± SD. Experiments were performed in triplicate. The analysis demonstrated a significant concentration effect ($p < 0.001$). Gentamicin, 10 µg/disc, was used as the positive control.

Table 4. MIC and MBC values of T. vulgaris absolute

Organism	MIC (µL/mL)	MBC (µL/mL)	MBC/MIC modal ratio
MRSA	2.5	5.0	2
MSSA	1.25	2.5	2
E. coli ESBL	5.0	10.0	2
K. pneumoniae ESBL	5.0	10.0	2
P. aeruginosa	10.0	20.0	2
P. mirabilis	2.5	5.0	2
C. freundii	5.0	10.0	2
E. aerogenes	5.0	10.0	2
S. flexneri	2.5	5.0	2
S. dysenteriae	2.5	5.0	2

Discussion

The present study demonstrated that low-temperature solvent extraction produced a T. vulgaris absolute with a yield of 1.92 ± 0.12%, compared with 0.85 ± 0.08% obtained by steam distillation. This difference is expected because the two extraction procedures operate according to different principles and produce chemically distinct preparations. The absolute should therefore not be considered simply a higher-yielding form of the essential oil [3,4].

The GC-MS profile showed thymol and carvacrol as the predominant constituents, accounting for 42.5% and 28.3%, respectively. Together, these compounds represented more than 70% of the identified chromatographic profile. Thymol and carvacrol have been shown experimentally to affect bacterial membrane integrity and permeability, providing a plausible explanation for part of the antibacterial activity observed in the present study [10,14,15].

The presence of γ-terpinene (6.8%) and p-cymene (5.2%) is also noteworthy. Although these compounds may exhibit weaker antibacterial effects than phenolic monoterpenes when evaluated individually, their presence in complex plant preparations may contribute to the overall biological activity. Interactions among constituents are one reason why the activity of a whole plant preparation cannot always be predicted from the concentration of a single compound [11,16].

The absolute demonstrated a clear concentration-dependent increase in inhibition-zone diameter. At 50%, the largest inhibition zone was observed against MSSA, followed by P. mirabilis and MRSA, whereas P. aeruginosa showed the smallest inhibition zones among the tested organisms.

The relatively lower susceptibility of Gram-negative bacteria may be related to the outer membrane, which

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provides an additional permeability barrier to hydrophobic compounds. P. aeruginosa also possesses several intrinsic resistance mechanisms, including restricted outer-membrane permeability and multidrug efflux systems [17,18]. Nevertheless, susceptibility was species-dependent, as demonstrated by the relatively strong response of P. mirabilis.

The MIC findings were consistent with the disc diffusion results. MSSA had the lowest MIC (1.25 µL/mL), whereas P. aeruginosa had the highest MIC (10 µL/mL). These findings suggest that the antibacterial effect of the preparation varies substantially among bacterial species.

The MBC/MIC ratio was ≤4 for the organisms tested, with a modal ratio of 2. A ratio of ≤4 is commonly used as an indicator consistent with bactericidal activity in vitro [19]. Accordingly, the results support a predominantly bactericidal effect under the experimental conditions used. This interpretation is restricted to the in-vitro assay and should not be extrapolated to clinical efficacy.

An important feature of the study was the inclusion of clinical isolates and resistant phenotypes. Approximately 40% of the S. aureus isolates were classified as MRSA, while approximately 35% of E. coli and K. pneumoniae isolates were ESBL producers. Overall, 24.8% of the isolates met the predefined MDR criterion. The observed activity against resistant isolates is of interest because it suggests that plant-derived preparations may retain antibacterial activity against organisms that show resistance to conventional antimicrobial agents. However, the mechanisms responsible for this activity were not investigated in the present study.

The MIC values obtained here are within the range reported in previous investigations of thyme preparations, although direct comparisons should be made cautiously because essential oils and absolutes are chemically different products and because bacterial strains, assay conditions, and inoculum preparation may differ between studies [20,21].

The present findings also emphasize the importance of distinguishing an absolute from an essential oil. The solvent-based procedure used here may produce a chemical profile that differs from a steam-distilled product. Consequently, biological findings reported for thyme essential oils cannot automatically be extrapolated to the absolute examined in this study.

The study has several limitations. First, the experiments were entirely in vitro, and no conclusions regarding clinical efficacy or therapeutic safety can be drawn. Second, cytotoxicity was not assessed. Third, antibiofilm activity and interactions with conventional antibiotics were not investigated. Fourth, long-term chemical stability was not evaluated. Finally, the GC-MS analysis was based primarily on spectral-library and retention-index comparisons; authenticated reference standards and quantitative validation would strengthen the identification of individual compounds.

Despite these limitations, the study provides preliminary evidence that a laboratory-prepared T. vulgaris absolute obtained from Libyan plant material possesses antibacterial activity against clinically relevant bacterial isolates, including MRSA and ESBL-producing strains.

Conclusion

Low-temperature solvent extraction produced a Thymus vulgaris L. absolute with a yield of 1.92 ± 0.12% (w/w), compared with 0.85 ± 0.08% for the steam-distilled essential oil. GC-MS analysis identified thymol and carvacrol as the predominant constituents, accounting for 42.5 ± 1.8% and 28.3 ± 1.5%, respectively.

The prepared absolute exhibited concentration-dependent antibacterial activity against the tested clinical isolates. MIC values ranged from 1.25 to 10 µL/mL, while MBC values ranged from 2.5 to 20 µL/mL. The MBC/MIC ratio was ≤4, with a modal value of 2, supporting a predominantly bactericidal effect under the conditions of the in-vitro assay.

The findings provide preliminary evidence supporting further investigation of T. vulgaris absolute as a source of antibacterial compounds. However, therapeutic use cannot be recommended on the basis of these findings alone. Cytotoxicity, antibiofilm activity, chemical stability, mechanism of action, antimicrobial combinations, and appropriately designed in-vivo safety and efficacy studies are required before clinical applications can be considered.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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