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The Effect of Extraction Methods of *Cassia alata* L. Leaves on Flavonoid, Phenolic Contents, and Antifungal Activity

Pengaruh Metode Ekstraksi Daun Ketepeng (*Cassia alata* L.) terhadap Kadar Flavonoid, Kadar Fenol, dan Aktivitas Antijamur

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ABSTRACT

Cassia alata L. is used traditionally to treat fungal diseases such as tinea pedis (athlete's foot), caused by *Trichophyton rubrum*. This study examined how three extraction methods (maceration, reflux, and ultrasonication) applied to *C. alata* leaves affect total flavonoid content (TFC), total phenolic content (TPC), and antifungal activity. TFC and TPC were determined by UV–Vis spectrophotometry, and antifungal activity was assessed by the well diffusion method. Ultrasonication produced the highest TFC (4.68 mg QE/g), TPC (8.92 mg GAE/g), and antifungal activity (inhibition zone diameter 25.7 mm) compared with maceration and reflux. Ultrasonication was therefore more effective than maceration and reflux at extracting the constituents of *C. alata* leaves that act against *T. rubrum*.

ABSTRAK

Daun ketepeng (*Cassia alata* L.) banyak digunakan secara empiris untuk mengobati penyakit jamur, termasuk tinea pedis (kutu air) yang disebabkan oleh *Trichophyton rubrum*. Penelitian ini bertujuan meneliti pengaruh tiga metode ekstraksi (maserasi, refluks, dan ultrasonikasi) daun ketepeng terhadap kadar flavonoid total (TFC), kadar fenol total (TPC), dan aktivitas antijamur. TFC dan TPC diukur dengan spektrofotometri UV–Vis, sedangkan aktivitas antijamur diuji dengan metode difusi sumuran. Ultrasonikasi menghasilkan TFC tertinggi (4,68 mg QE/g), TPC tertinggi (8,92 mg GAE/g), dan aktivitas antijamur tertinggi (diameter zona hambat 25,7 mm) dibandingkan maserasi dan refluks. Dengan demikian, ultrasonikasi lebih efektif dibandingkan maserasi dan refluks dalam mengekstrak senyawa aktif daun *C. alata* yang berkhasiat terhadap *T. rubrum*.

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1. INTRODUCTION

Tinea pedis, commonly referred to as athlete's foot, is an infection caused by dermatophyte fungi that affects the skin between the toes, the soles, and the lateral parts of the feet (Silva et al., 2022). Typical symptoms include peeling skin, itching, discharge, and an unpleasant odor. *Trichophyton rubrum*, the species most often responsible, is anthropophilic and spreads between humans by direct contact or through an intermediary (Piraccini & Alessandrini, 2015). Antifungal medicines for tinea pedis are given orally, topically, or as combination therapy (Warouw et al., 2021). Inappropriate or prolonged use of these agents, however, can cause adverse effects and resistance.

In Indonesia, the leaves of the candle bush, *Senna alata* (L.) Roxb., also known as *Cassia alata* L., are widely used in traditional medicine for skin disorders (Lathifah et al., 2021). The species is recognized for its antimicrobial properties and has been used against ringworm, tinea versicolor, scabies, malaria, constipation, suppurative skin inflammation, syphilis, herpes, influenza, and bronchitis (Oladeji et al., 2020; Sesa et al., 2014). An earlier study using the disk diffusion method reported that *C. alata* extract inhibited *T. rubrum* with an inhibition zone diameter of 14 mm, as well as inhibiting *Candida albicans* (Lathifah et al., 2021).

C. alata contains bioactive constituents including flavonoids and phenolics (James et al., 2022; Oladeji et al., 2020). No study, however, has compared extraction methods for recovering flavonoids and phenolics from *C. alata* or related the recovery to antifungal activity. This study therefore aimed to determine the effect of three extraction methods (maceration, reflux, and ultrasonication) on the total flavonoid content (TFC), total phenolic content (TPC), and antifungal activity of *C. alata* leaf extract against *T. rubrum*.

2. METHODS

2.1. Materials and Equipment

The laboratory tools were analytical balance (Ohaus, Troy Hills, USA), oven (Binder, Tuttlingen, Germany), reflux apparatus (Iwaki, Tokyo, Japan), rotary evaporator (Heidolph, Schwabach, Germany), UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan), ultrasonicator bath 3800® (Branson, Brookfield, USA), autoclave (GEA, Dusseldorf, Germany), biosafety cabinet (Monmouth Scientific Limited, Bridgwater, UK), and waterbath (Memmert GmBH, Schwabach, Germany).

The leaves of *C. alata* were harvested in Bantul, Special Region of Yogyakarta, Indonesia. *Trichophyton rubrum* ATCC 28188 was obtained from Thermo Fisher Scientific (Kansas, USA). Sabouraud dextrose agar (SDA) medium was from HiMedia (Mumbai, India), 2% ketoconazole lotion (DermaXP®) from Jakarta, Indonesia, and gallic acid and aluminum chloride ($AlCl_3$) from Sigma-Aldrich (St. Louis, USA). Dimethyl sulfoxide (DMSO), sodium chloride (NaCl), sodium acetate (CH_3COONa), quercetin, Folin–Ciocalteu reagent, sodium hydroxide (NaOH), methanol, and 96% ethanol were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

2.2. Preparation of the *C. alata* Extract

Maceration: 22 g of *C. alata* leaf powder was soaked in 96% ethanol at a 1:5 ratio (w/v). After 24 h the macerate was filtered. The extraction was performed as three independent replicates (Indonesian Ministry of Health, 2022).

Reflux: 22 g of *C. alata* leaf powder was placed in a round-bottom flask with 96% ethanol at a 1:5 ratio (w/v), and extraction was carried out for 3 h. The extraction was performed in three replicates.

Ultrasonication: 22 g of *C. alata* leaf powder was placed in an Erlenmeyer flask, and 110 mL of 96% ethanol was added (1:5 ratio, w/v). The mixture was sonicated for 30 min at room temperature. The extraction was performed in three replicates.

The filtrates obtained after extraction were concentrated on a rotary evaporator, the mass of the concentrated extract was recorded, and the yield was calculated (Nursamsiar, 2022).

2.3. Total Flavonoid Content of the *C. alata* Extract

TFC was measured following the Indonesian Herbal Pharmacopoeia (Indonesian Ministry of Health, 2022). A 0.5 mL aliquot of the test solution was mixed with 1.5 mL of 96% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of water. The mixture was incubated for 30 min at room temperature, and absorbance was read at 435 nm. Quercetin served as the standard, and TFC was expressed as milligrams of quercetin equivalent (QE) per gram of dry weight. The measurement was performed in triplicate.

2.4. Total Phenolic Content of the *C. alata* Extract

TPC was measured following the Indonesian Herbal Pharmacopoeia (Indonesian Ministry of Health, 2022). One milliliter of the test solution and 5.0 mL of Folin–Ciocalteu reagent were combined in a test tube and incubated for 8 min. Then 4.0 mL of 1% NaOH was added and the mixture was incubated for 1 h. Absorbance was read at 730 nm. Gallic acid was used for the standard curve, and TPC was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight. The measurement was performed in triplicate.

2.5. Antifungal Assay by the Well Diffusion Method

The *T. rubrum* inoculum was prepared following the plate-inoculation procedure of CLSI document M44-A2 (Clinical and Laboratory Standards Institute [CLSI], 2009). Because that document covers disk diffusion testing of yeasts, two modifications were made for this filamentous dermatophyte: the extracts were applied in wells cut into the agar rather than on paper disks, and the incubation was adapted to the growth of *T. rubrum*. A cotton swab was dipped in the *T. rubrum* suspension adjusted to the 0.5 McFarland standard, and the suspension was spread over the surface of the SDA medium. Five wells were cut in the medium and filled with 50 µL of 10% *C. alata* extract prepared by

maceration, reflux, or ultrasonication. The negative control was 10% DMSO and the positive control 2% ketoconazole. After incubation at room temperature for six days, the diameter of the inhibition zone was measured with a ruler.

2.6. Statistical Analysis

Yield, TFC, TPC, and inhibition zone diameter were each measured in three independent replicates and are reported as mean ± standard deviation. Normality was checked with the Shapiro–Wilk test and homogeneity of variance with Levene's test; both held for every data set, so group means were compared by one-way analysis of variance followed by Tukey's honestly significant difference test for all pairwise comparisons, at a significance level of 0.05. The relationships between TFC and TPC and between TFC and inhibition zone diameter were quantified with Pearson's correlation coefficient computed on the nine individual replicate pairs (three replicates × three extraction methods). Analyses were carried out in Python 3.11 with SciPy 1.17.1 and statsmodels 0.15.0.

3. RESULTS AND DISCUSSION

Selecting the extraction method is essential for maximizing bioactive content and biological activity. Three methods were compared for *C. alata*: maceration, reflux, and ultrasonication, chosen to represent conventional and non-conventional extraction principles — cold extraction, hot extraction, and high-frequency sound waves, respectively (BPOM RI, 2023; Zhang et al., 2023). Ethanol 96% was used as the solvent. Ethanol is semi-polar, with a polarity index of 5.2, and therefore dissolves flavonoid and phenolic compounds (Adriana et al., 2024; Sadek, 2002); it extracts efficiently, is self-preserving, and is relatively safe (Ahmed et al., 2026). It is also volatile, which facilitates evaporation to a concentrated extract, and it is widely used in pharmaceuticals, herbal medicine development, natural product extraction, and the food and beverage industry (Awad et al., 2021).

3.1. Extraction Yield

Extraction yield indicates the percentage of extract obtained from the plant material and thus the efficiency of the process. A higher yield points to a more efficient extraction and a greater likelihood that the extract contains more active compounds.

Ultrasonication gave the highest yield, followed by reflux and maceration (**Table 1**). Ultrasonic waves accelerate solvent penetration into plant tissue, so larger quantities of secondary metabolites are released: high-frequency waves reaching the sample surface strike particles at high speed and drive active compounds out of the cells and into the ethanol (Avif & Yaqhsa, 2021; Yusoff et al., 2022). The same ranking has been reported previously, with ultrasonic extraction giving the highest yield, followed by reflux and maceration (Tinasy & Wijayati, 2024; Yusnira & Ediputra, 2025). Ultrasonication is also the fastest of the three: maceration usually takes several days and reflux several hours, whereas ultrasonication takes minutes.

Table 1. Yield of the *C. alata* extract obtained by three extraction methods.

Extraction Method	Yield (%)
Maceration	7.60 ± 0.687 ^{x,z}
Reflux	9.41 ± 0.193 ^{x,y}
Ultrasonication	11.91 ± 0.562 ^{y,z}

Note. Values are mean ± SD (n = 3). Superscripts mark significant differences by one-way ANOVA with Tukey's HSD test: x, maceration vs. reflux ($p = 0.013$); y, reflux vs. ultrasonication ($p = 0.003$); z, maceration vs. ultrasonication ($p < 0.001$). HSD, honestly significant difference; SD, standard deviation.

3.2. Total Flavonoid and Total Phenolic Contents

The extraction method also influenced TFC and TPC. **Figure 1** and **Figure 2** show that the extract obtained by ultrasonication contained 4.68 mg QE/g and 8.92 mg GAE/g, respectively, significantly higher than the extracts obtained by maceration and reflux ($p < 0.001$). A comparable pattern has been reported for black pepper, where ultrasonic extraction gave the highest TPC among the techniques tested (Milenković et al., 2025). When ultrasonic waves enter the extraction medium they generate small bubbles by cavitation; the collapse of these bubbles converts wave energy into kinetic energy, producing heat and shear forces that break down the plant cell wall and let the solvent enter more easily, which raises the extraction rate substantially (Shen et al., 2023). Phenolic and flavonoid constituents therefore diffuse into the solvent more rapidly.

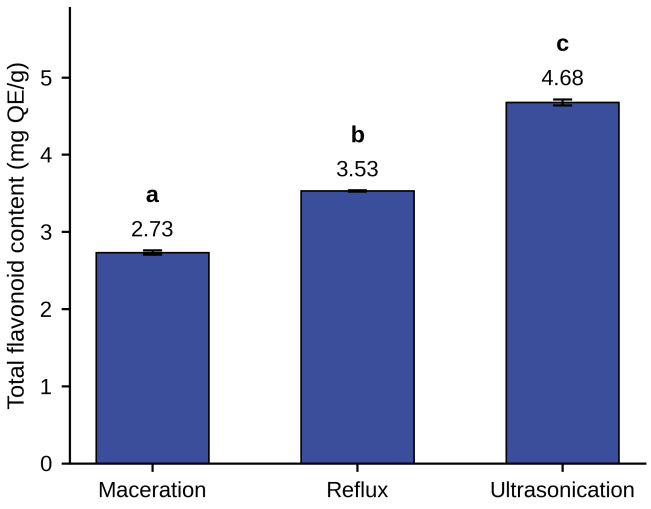

Figure 1. Total flavonoid content (TFC) of the *C. alata* extract obtained by maceration, reflux, and ultrasonication. Bars are mean ± SD (n = 3); different letters indicate significant differences (one-way ANOVA with Tukey's HSD test, $p < 0.001$).

3.3. Antifungal Activity

The extraction method also affected the activity of the *C. alata* extract against *T. rubrum* (**Table 2**). Extracts obtained by ultrasonication were the most active, followed by reflux and maceration, although all three remained less active than ketoconazole. Ketoconazole is widely used against skin infections such as ringworm, athlete's foot, and tinea versicolor; it inhibits

the cytochrome P450 enzyme 14α-demethylase (CYP51), thereby blocking ergosterol biosynthesis and compromising the integrity of the fungal cell membrane (Singh et al., 2023).

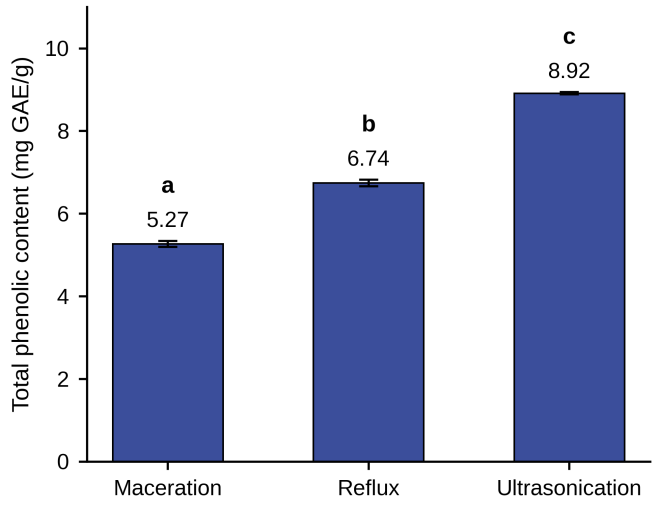

Figure 2. Total phenolic content (TPC) of the *C. alata* extract obtained by maceration, reflux, and ultrasonication. Bars are mean ± SD (n = 3); different letters indicate significant differences (one-way ANOVA with Tukey's HSD test, $p < 0.001$).

Table 2. Antifungal activity of the *C. alata* extract against *T. rubrum*.

Extraction Method	Inhibition zone diameter (mm)	Category
Maceration	5.3 ± 1.53 ^{a,c,d,e}	Weak
Reflux	18.0 ± 1.00 ^{a,b,d,e}	Medium
Ultrasonication	25.7 ± 2.52 ^{b,c,d,e}	Strong
Ketoconazole	30.0 ± 1.00 ^{d,f}	Strong
10% DMSO	0 ^{e,f}	-

Note. Inhibition zone diameters are mean ± SD (n = 3) in millimetres. Superscripts mark significant differences by one-way ANOVA with Tukey's HSD test: a, maceration vs. reflux ($p < 0.001$); b, reflux vs. ultrasonication ($p < 0.001$); c, maceration vs. ultrasonication ($p < 0.001$); d, maceration, reflux, and ultrasonication vs. ketoconazole ($p < 0.001$, $p < 0.001$, and $p = 0.029$, respectively); e, maceration, reflux, and ultrasonication vs. 10% DMSO ($p = 0.008$, $p < 0.001$, and $p < 0.001$, respectively); f, ketoconazole vs. 10% DMSO ($p < 0.001$). DMSO, dimethyl sulfoxide; HSD, honestly significant difference; SD, standard deviation.

The macerated extract gave the lowest yield, TFC, TPC, and antifungal activity. Maceration extracts compounds by soaking plant material in a solvent at room temperature (Mondal et al., 2026). The solvent penetrates the tissue, the cell walls soften and swell, and soluble constituents dissolve according to the principle that like dissolves like; the compounds then diffuse out of the cells along the concentration gradient until equilibrium is reached. Maceration is simple and reasonably effective but slow, and it yields relatively little (Zhang et al., 2023).

Reflux gave higher TFC, TPC, and antifungal activity than maceration but lower values than ultrasonication. Reflux uses volatile organic solvents in a cycle of heating, evaporation, condensation, and soaking. The heat increases the solubility, diffusivity, and mass transfer of compounds within the plant matrix, which favors the extraction of phenolics and other secondary metabolites (Haideri et al., 2024). Although this improves efficiency and limits solvent use, prolonged heating makes the method unsuitable for heat-sensitive materials (Travis et al., 2026), and some heat-sensitive compounds may degrade. This may explain why TFC, TPC, and antifungal activity after reflux were lower than after ultrasonication, a pattern consistent with reports that reflux is less efficient than modern techniques (Wen et al., 2018). That reflux nevertheless outperformed maceration on every measure suggests that heating released more extractable compounds than it degraded.

Figure 3 shows a positive correlation between TFC and TPC ($r = 0.9992$, $n = 9$, $p < 0.001$) and between TFC and antifungal activity ($r = 0.9595$, $n = 9$, $p < 0.001$). The near-perfect correlation between TFC and TPC is chemically expected, because flavonoids are themselves polyphenolic compounds, so a rise in flavonoid content raises the measured TPC response. The correlation between TFC and antifungal activity suggests that flavonoids are among the metabolite groups contributing most to the activity of the extract. Flavonoids reported in *C. alata* include kaempferol, kaempferol-3-O-β-D-glucopyranoside, 3,5,7,4′-tetrahydroxyflavone, and 2,5,7,4′-tetrahydroxyisoflavone (Fatmawati et al., 2020), as well as naringenin, quercetin, apigenin, kaempferol, and luteolin (Yagi et al., 2025). Phenolics reported in the species include dihydroxybenzoic acid, p-coumaric acid, sinapic acid, and ferulic acid (Omer et al., 2022) , which may also contribute to antifungal activity. A separate study identified eight constituents of *C. alata* active against fungal and bacterial plant pathogens: rhein, aloe-emodin, aloe-emodin-8-O-β-D-glucoside, kaempferol, kaempferol-3-O-glycoside, kaempferol-3-O-gentiobioside, epiafzelechin, and methyl 2,4,6-trihydroxybenzoate (Pham et al., 2021). Because the present study did not identify individual constituents, further work on the chemical composition of the extract and the contribution of each compound to the activity is needed.

Flavonoids and phenolics act against fungi through several mechanisms, including damage to and denaturation of fungal cells. Flavonoids can interact with the cell wall and cause cell death (Lathifah et al., 2021), and those carrying hydroxyl groups interact directly with membrane lipids, altering permeability, damaging membrane structure, and reducing viability and growth (Lee & Kim, 2022). Flavonoids in the *C. alata* extract may also disrupt membrane proteins involved in transport and energy production, inhibiting the growth of *T. rubrum* (Tang et al., 2025). Phenolics likewise damage the fungal cell membrane and increase its permeability, destabilizing the cell and inhibiting growth. Phenolic acids have been reported to reduce biofilm formation and phospholipase production and to inhibit virulence factors such as the yeast-to-hypha transition (Possamai Rossatto et al., 2021). Eugenol, another phenolic compound, disrupts enzymes of ergosterol biosynthesis — ergosterol being essential to the

integrity and permeability of the fungal membrane — and also reduces keratinase activity and suppresses SUB3 gene expression (Aliabasi et al., 2023). Effects of this kind on fungal virulence at the cellular and molecular level may explain the inhibition of *T. rubrum* observed here.

This study has several limitations. Only one solvent (96% ethanol) was tested; only one reference strain (*T. rubrum* ATCC 28188) was used rather than clinical isolates; the constituents of the extract were not identified at the compound level; and only one extract concentration (10%) was assayed. No dose–response, MIC, or MFC data are available to support the conclusion that the extract is more effective.

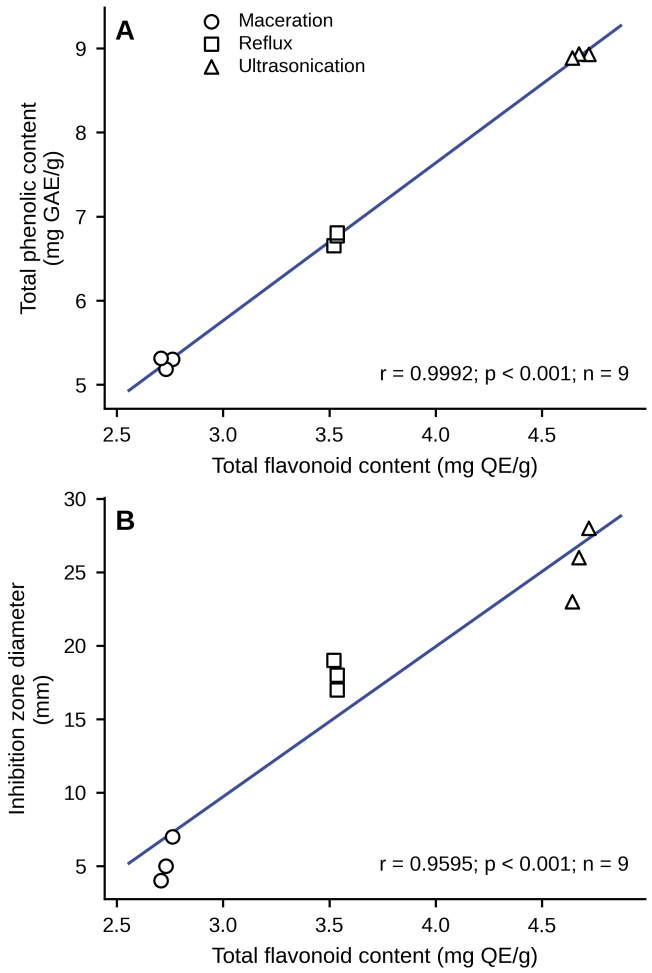

Figure 3. Correlation between total flavonoid content and total phenolic content (A) and between total flavonoid content and inhibition zone diameter (B) for the *C. alata* extract obtained by maceration, reflux, and ultrasonication. Each point is one extraction replicate (n = 9); the line is the least-squares fit.

4. CONCLUSION

Ultrasonication was the most effective of the three methods for extracting *C. alata* leaves, giving the highest yield, TFC, TPC, and antifungal activity against *T. rubrum* in the shortest extraction time. The extract is therefore a promising plant-derived candidate for further study against dermatophyte fungi, although compound-level identification and dose–response testing are still required.

AUTHOR CONTRIBUTIONS

Conceptualization, T.R. and S.M.; methodology, S.M.; software, T.R. and S.M.; validation, H.S.; formal analysis, T.R.; investigation, T.R.; resources, S.M.; data curation, T.R.; writing—original draft preparation, T.R.; writing—review and editing, S.M.; visualization, S.M.; supervision, H.S.; project administration, T.R. All authors have read and agreed to the published version of the manuscript.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable. This study did not involve human participants or animal subjects.

INFORMED CONSENT STATEMENT

Not applicable. This study did not involve human participants.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ROLE OF FUNDERS

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DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) USE

The author(s) declare that no generative AI or AI-assisted technologies were used in the preparation or writing of this manuscript. All contents were produced entirely by the author(s) without any automated assistance.

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