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Research Article

Comparative Evaluation of Synergistic Antibacterial Effects in Blended Essential Oil against Gram-Positive and Gram-Negative Pathogens

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Abstract: The rising emergence of multidrug-resistant bacteria has heightened the search for novel antibiotic agents. Essential Oils (EOs) are complex combinations of volatile organic compounds generated by plants, with many exhibiting significant antimicrobial properties. This study evaluates the antibacterial movement of single and blended EOs against representative gram-positive bacteria *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* (MRSA) and gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. Using the disk diffusion method, blended EOs exhibited greater inhibition zones than those of single EOs, with gram-positive bacteria (*S. aureus* and MRSA) being more susceptible than gram-negative bacteria. Notably, *P. aeruginosa* demonstrated complete resistance, likely due to its complex outer membrane. The Minimum Inhibitory Concentration (MIC) was decided via micro-well dilution assay in line with the Clinical and Laboratory Standards Institute criteria, further validating the enhanced efficacy of blended EOs. Synergistic interactions among active components in the EOs likely contributed to the lower MIC values observed for blended EOs. This study highlights the blended EOs as essential antibacterial agents, particularly against gram-positive pathogens such as MRSA. However, resistance in gram-negative bacteria highlights the need for targeted formulation strategies. Future research should focus on evaluating EO efficacy against diverse multi-drug resistance pathogens, optimizing EO formulations for clinical practice. These results support the development of EO-based therapies as sustainable alternatives to synthetic antimicrobials in combating multi-drug resistant infections. The study establishes the framework for incorporating EOs into antimicrobial strategies, addressing the crucial need for novel solutions to the global antibiotic resistance challenge.

Keywords: Essential Oils, Anti-Bacterial Agents, Gram-Positive Bacteria, Gram-Negative Bacteria, Drug Resistance, Natural

Introduction

Essential Oils (EOs) are highly unstable substances obtained from discrete parts of plants, traditionally used in medicine for biological activities and antibacterial, non-steroidal drug, antioxidant, and antifungal properties (Beyki *et al.*, 2014). Although much research has highlighted that the antibacterial and antioxidant properties of EOs are their primary biological activities, other potential effects remain insufficiently investigated. In particular, the transmission of antibiotic-resistant bacteria is causing serious morbidity and mortality worldwide and effective alternatives are urgently needed (Tacconelli *et al.*, 2018). Blends of essential oils offer the potential to complement the limitations of individual EOs and provide natural alternatives to synthetic antibacterial agents (Mutlu-Ingok *et al.*, 2019). In addition, a key characteristic of EOs and their elements is their hydrophobic nature, allowing them to enter the lipid layers of bacterial cell membranes and mitochondria, which damages and increases permeability (Burt, 2004). The biological effects of EOs are determined not only by their main components but also by minor constituents that enhance their overall activity (Calo *et al.*, 2015). Various factors, including plant kinds, geographic foundation, ecological conditions, maturity stage, and extraction techniques, play crucial roles in shaping the chemical composition of EOs (Nazzaro *et*

al., 2017). Consequently, strategies to enhance the consistency and activity of EOs are essential. Technologies like encapsulation and active packaging present promising solutions to improve their performance and application (Prakash and Kiran, 2016). Although EOs have demonstrated antibacterial activity against various pathogens, systematic evaluations of blended EO efficacy and their synergistic effects are limited. Blended EOs, composed of two or more individual oils, leverage the unique chemical components and mechanisms of each oil to achieve a broader spectrum of antibacterial activity. This synergistic approach not only enhances antibacterial potency but also minimizes potential side effects and expands the range of target pathogens (Hammer *et al.*, 1999; Bassolé and Juliani, 2012). The goal of this study is to systematically estimate the antibacterial influence of blended EOs, hypothesizing that their combined use can yield stronger effects than single oils by employing the Clinical and Laboratory Standards Institute (CLSI). A globally recognized method for assessing antibacterial efficacy—this study ensures the reliability and reproducibility of its findings. The investigation focuses on the ability of blended EOs to combat various pathogenic microorganisms, addressing the urgent need for alternative treatments in light of rising resistance to synthetic antibacterial agents (Nostro and Papalia, 2012; Langeveld *et al.*, 2013). Ultimately, this study seeks to demonstrate the potential of blended EOs as natural antibacterial agents, offering an innovative and sustainable approach to addressing the growing issue of antibacterial resistance.

Materials and Methods

Essential Oils

In previous studies, bergamot, orange, peppermint, and rosemary are representative examples of plants with antimicrobial activity (Bakkali *et al.*, 2008). In this study, four single EOs (Bergamot (S1), Peppermint (S2), Rosemary (S3), Orange (S4)) and four blended EOs (Relax Aroma Oil (B1), Refreshing Aroma Oil (B2), Zest Aroma Oil (B3), Revitalizing Aroma Oil (B4)) were used. All EOs were 100% pure and commercially procured from the Herb Island Agricultural Cooperative, Pocheon-si, Korea. The experiment aimed to quantify the antibacterial effects of blended EOs, supplemented by Minimum Inhibitory Concentration (MIC) and disk diffusion method data. The primary components of each blended EO, as outlined in Table (1), include 45% Bergamot fruit oil in B1, 67% Peppermint leaf oil in B2, 26% Rosemary leaf oil in B3, and 48% Orange peel oil in B4. These combinations were specifically designed to explore the potential synergistic impact of the blended EOs on antibacterial activity.

Bergamot fruit oil, the predominant component of B1, is labeled as S1. Peppermint leaf oil, the primary component of B2, is labeled as S2. Rosemary leaf oil, the main component of B3, is labeled as S3. Orange peel oil, the dominant component of B4, is labeled as S4. Oils contributing less than 10% are categorized as “other oils.”

Table 1: Composition table showing the names and contents of the components of blending oils

Oils	Ingredients	Name	Percentage of compounds (%)
B1	1	Bergamot fruit oil	45
	2	Lavender flower oil	35
	3	Scented geranium oil	10
	4	other oils	10
B2	1	Peppermint leaf oil	67
	2	Lavender flower oil	10
	3	Pine needle oil	10
	4	other oils	13
B3	1	Rosemary leaf oil	26
	2	Scented geranium flower oil	20
	3	Fennel oil	15
	4	Pine berry oil	15
	5	Bergamot fruit oil	11
	6	other oils	13
B4	1	Orange peel oil	48
	2	Bergamot fruit oil	20
	3	Scented geranium oil	16
	4	Lavender flower oil	14
	5	other oils	2

Microorganisms and Culture Conditions

The following microorganisms were used to estimate the antibacterial effects: *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *S. aureus* (MRSA, ATCC 33591), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). For the MIC determination, *the S. aureus* (ATCC 29213) strain was utilized. Bacteria were grown on Mueller–Hinton Agar (MHA) at 37°C for 24 h. After incubation, bacterial suspensions were prepared by diluting the cultures in physiological saline and changing them to a 0.5 McFarland (approximately 1.5×10^8 CFU/mL) using a density turbidimeter (bioMérieux, Marcy-l’Étoile, France).

Disk Diffusion Assay

Bacterial suspensions prepared on MHA were evenly spread using a sterile cotton swab. Each 6-mm sterile disk was loaded with 20 µL of EO, dried at 21°C for 20 min, and placed onto the agar surface. Unless otherwise stated, EOs were tested at 100% concentration (undiluted). Positive controls were validated using antimicrobial disks compliant with Tier 1 and 2 standards for each organism, following the CLSI M100 ED34: 2024 guidelines. All plates were protected at 37°C for 24 h. The antibacterial effect was determined by calculating the diameter of the inhibition zones (in mm) surrounding

each disk. Experiments were done in triplicate and the outcomes were reported as mean ± standard deviation.

MIC

The MIC is assessed by the liquid medium microdilution scheme. EOs were serially diluted in Mueller–Hinton Broth (MHB) using a two-fold dilution method, with sterile distilled water as the solvent. Bacterial suspensions were organized in MHB at a concentration of 5×10^6 CFU/mL. A 200 µL bacterial suspension was used as the positive growth control, while 200 µL of MHB was utilized as the negative control. The inoculated 96-well plates are protected at 35°C for 18-24 h under aerobic conditions. The MIC was identified as the lowest concentration at which no bacteria appeared. Absorbance at 600 nm was measured with a spectrophotometer (e-innotech, Daejeon, Korea) to validate the results. Antibacterial agents were serially diluted from 128 µg/mL in two-fold steps using a sterile pipette for quality control, following the CLSI M100 ED34: 2024 guidelines. Statistical analysis was conducted utilizing Student's t-test, with p-values less than 0.05 as statistically significant. Data were estimated by SPSS version 26. The MIC of B1 (25 µg/mL) against *S. aureus* and MRSA is significantly lower ($p<0.05$) than that of S1 (50 µg/mL), indicating that the blended EO exhibited greater antibacterial activity compared to the single EO.

Results

Antibacterial Effect of EOs by Disk Diffusion Method

The antibacterial movement of blended and single EOs was assessed against *E. coli*, *P. aeruginosa*, *S. aureus*, and MRSA using the disk diffusion technique. Comparative analysis of the reserve zones revealed that blended EOs tended to exhibit greater antibacterial activity than single EOs. As shown in Table (2), the reserve zones for blended EO B1 against *S. aureus* and MRSA were 19.6±0.2 mm and 18.4±0.2 mm, respectively, which were significantly larger than those for single EOs S1 and S2 ($p<0.05$).

The study further demonstrated that gram-positive bacteria, like *S. aureus* and MRSA, were more susceptible to the antibacterial effects of EOs than gram-negative bacteria like *E. coli*. Notably, no inhibition zone was observed for *P. aeruginosa*, indicating resistance to both blended and single EOs.

Table 2: Antimicrobial activity of essential oils determined by disc diffusion method against S. aureus (ATCC 25923), MRSA (ATCC 33591), E. coli ATCC 25922 and P. aeruginosa ATCC 27853

Bacteria	Inhibition Zone (mm)							
	S1	S2	S3	S4	B1	B2	B3	B4
S. aureus	11.2±0.2	15.2±0.1	13.2±0.1	ND	19.6±0.2	20.2±0.2	12±0.1	17.2±0.1
MRSA	11.2±0.1	14.8±0.1	7±0.1	ND	18.4±0.2	16±0.1	13.1±0.1	16.6±0.2
E. coli	9.1±0.07	10.1±0.07	10.2±0.07	ND	9.7±0.07	11±0.07	10.7±0.06	13.1±0.1
P. aeruginosa	ND	ND	ND	ND	ND	ND	ND	ND

Abbreviations: S. aureus, Staphylococcus aureus; MRSA, methicillin-resistant S. aureus; E. coli, Escherichia coli; P. aeruginosa, Pseudomonas aeruginosa; ND, not detected; Bergamot oil (S1), Peppermint oil (S2), Rosemary oil (S3), Orange oil (S4), Relaxation Aroma Oil (B1), Refreshing Aroma Oil (B2), Zest Aroma Oil (B3), Revitalizing Aroma Oil (B4)

Table 3: Minimum inhibitory concentration (MIC) of essential oils determined by broth dilution method against S. aureus (ATCC 25923), MRSA (ATCC 33591), E. coli ATCC 25922, P. aeruginosa ATCC 27853

Bacteria	Minimum inhibitory concentration percent of essential oils (%)	MIC (µg/mL)									
	S1	S2	S3	S4	B1	B2	B3	B4	Oxacillin	Vancomycin	Ampicillin	Ciprofloxacin
S. aureus	50	12.5	25	ND	25	25	100	100	0.5	2	X	X
MRSA	50	25	100	ND	25	50	100	50	ND	2	X	X
E. coli	100	100	100	ND	100	100	100	50	X	X	4	X
P. aeruginosa	ND	ND	ND	ND	ND	ND	ND	ND	X	X	X	1

Abbreviations: S. aureus, Staphylococcus aureus; MRSA, methicillin-resistant S. aureus; E. coli, Escherichia coli; P. aeruginosa, Pseudomonas aeruginosa; ND, not detected; X, Did not perform; Bergamot oil (S1), Peppermint oil (S2), Rosemary oil (S3), Orange oil (S4), Relaxation Aroma Oil (B1), Refreshing Aroma Oil (B2), Zest Aroma Oil (B3), Revitalizing Aroma Oil (B4) MIC values quantify the antimicrobial effects of essential oils, demonstrating the potential of bergamot, peppermint, rosemary and orange oils as natural antimicrobial agents against a variety of pathogens

MIC Evaluation by a Micro-Well Dilution Assay

The MIC of single essential oils and blended essential oils was found against gram-positive bacteria and gram-negative bacteria by the two-fold serial dilution method. Starting from 100% of the original EO solution, serial dilutions were prepared in MHB. The bacterial concentration was altered to 1.5×10^6 CFU/mL, with MHB serving as the negative control. Table (3) presents the MIC values observed visually 24 h

after inoculating the bacteria into 96-well plates. These results were further validated by assessing the absorbance of the bacterial culture delay at 600 nm (Figure 1). Lower MIC values indicated a stronger antibacterial effect. Blended EOs exhibited significantly lower MIC values than single EOs for mostbacterial strains, suggesting an enhanced antibacterial effect due to synergistic interactions among EO components. According to the CLSI guideline M100 ED34: 2024, MIC results for standard antimicrobial agents were used for quality control. *S. aureus* (ATCC 25923) was resistant to oxacillin (MIC: 0.12–0.5 µg/mL with a reference value of 0.5 µg/mL), *E. coli* (ATCC 25922) is resistant to ampicillin (MIC: 2–8 µg/mL with a reference importance of 4 µg/mL) and *P. aeruginosa* (ATCC 27853) is resistant to ciprofloxacin (MIC: 0.12–1 µg/mL with regards to significance of 1 µg/mL). These results confirmed the reliability of the MIC tests conducted in this study.

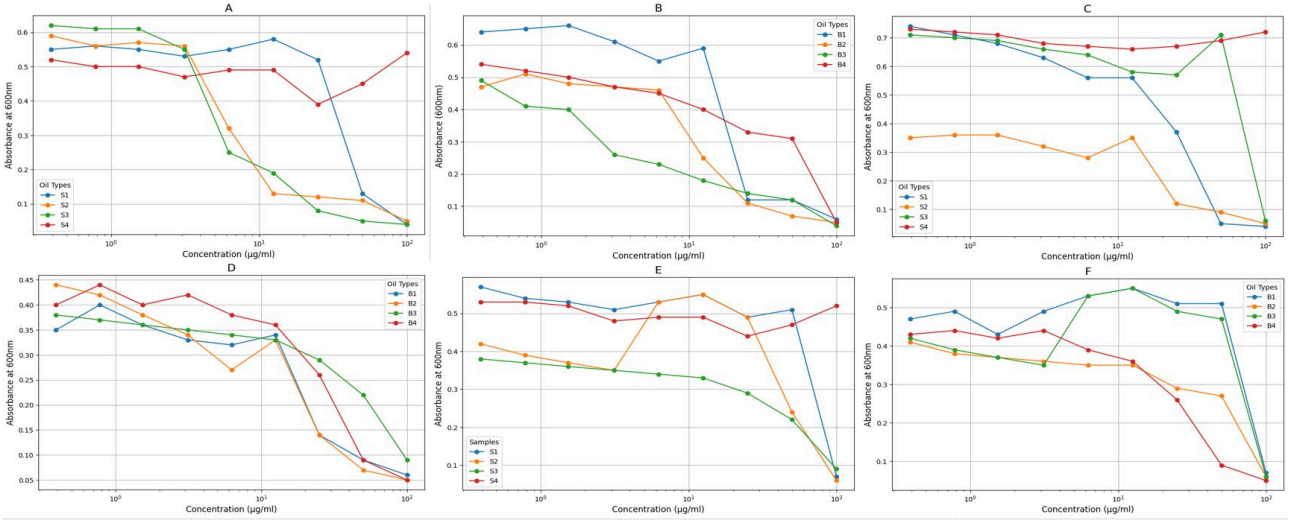

Fig. 1: MIC was measured by absorbance at 600 nm. For S. aureus (ATCC 25923), A: S1, S2, S3, S4, and B: B1, B2, B3, B4. For MRSA (ATCC 33591), C: S1, S2, S3, S4 and D: B1, B2, B3, B4. and for E. coli (ATCC 25922), E: S1, S2, S3, S4 and F: B1, B2, B3, B4; Abbreviations: S. aureus, Staphylococcus aureus; MRSA, methicillin-resistant S. aureus; E. coli, Escherichia coli; P. aeruginosa, Pseudomonas aeruginosa; Bergamot oil (S1); Peppermint oil (S2); Rosemary oil (S3); Orange oil (S4); positive control, pos; negative control, neg; Lower absorbance suggests stronger bacterial growth inhibition

Discussion

This study conducted a systematic evaluation of the antibacterial impact of blended EOs against S. aureus, MRSA, E. coli, and P. aeruginosa. These bacteria represent gram-positive, gram-negative microorganisms and major pathogens liable for various clinical infections (Trombetta *et al.*, 2005). *S. aureus* is identified to cause skin and soft tissue infections, pneumonia, and endocarditis and its treatment is becoming increasingly challenging due to the rise of resistant strains such as MRSA (Chambers and DeLeo, 2009). Pseudomonas species are well-known pathogens capable of producing infections, especially in immunocompromised persons, with the incidence of multidrug-resistant strains steadily increasing (Lambert, 2002). E. coli is a main cause of gastrointestinal and urinary tract infections and includes multiple resistant strains currently in circulation (Kaper *et al.*, 2004). In this study, P. aeruginosa, a gram-negative bacterium, exhibited no significant sensitivity to the antibacterial activity of EOs, while E. coli showed lower susceptibility compared to the gram-positive bacteria S. aureus and MRSA. EOs demonstrated stronger antibacterial effects against both S. aureus and MRSA than against the gram-negative strains. These results align with previous studies indicating that gram-positive bacteria are generally more susceptible to EOs compared to gram-negative bacteria (Zhang *et al.*, 2015) (Table 2). The varying susceptibility among gram-positive and gram-negative bacteria to EOs was well established. Gram-positive bacteria, such as S. aureus, typically display greater sensitivity to EOs compared to gram-negative bacteria. This variation is largely attributed to changes in the structural characteristics of their cell walls. The antibacterial effects of EOs are likely mediated through direct actions on the bacterial cell tissue, including disruption of skin, increased penetrability and the drip of vital cellular components such as potassium ions and protons, ultimately leading to cell death at specific concentrations (Del Carmen Beristain-Bauza *et al.*, 2019; Nikaido, 1994). Alterations in fatty acid composition, enhanced membrane fluidity, and the reserve of enzymatic activity or induction of cell lysis may also play roles in the antibacterial mechanisms of EOs. The cell membrane of gram-positive bacteria does not have the external membrane present in gram-negative bacteria, and is more permeable to hydrophobic molecules, allowing EOs to more readily penetrate and disrupt the bacterial membrane. Contrary to gram-negative bacteria take a more complex membrane

structure, containing of a lipid bilayer and an external membrane connected to the internal peptidoglycan layer by lipoproteins, which serves as a barrier to the entry of hydrophobic substances (Wang *et al.*, 2020; Cho *et al.*, 2020). Pseudomonas aeruginosa, a representative Gram-negative bacterium, highlights the challenge of overcoming resistance mechanisms of aeruginosa, particularly natural products such as EOs. Organisms typically resist antimicrobial agents through three primary mechanisms: Restricted uptake and active efflux, drug inactivation, and modification of the target site (Lambert, 2002). Major resistance mechanisms include efflux pump systems, biofilm formation, and reduced outer membrane permeability. Efflux pumps are important in the emergence of multidrug resistance in bacteria. Four multidrug efflux pumps in *P. aeruginosa* were responsible for expelling toxic molecules and reducing antibiotic susceptibility (Colclough *et al.*, 2020). Additionally, bacterial biofilms exhibit resistance to antibiotic treatments and immune system clearance. The use of antibiotics is often insufficient to eliminate biofilm infections due to adaptive resistance. Therefore, novel biofilm-specific therapies are needed (Taylor *et al.*, 2014).

The antibacterial activity of EOs varies depending on the chemical profile and the ratio of active ingredients (Bora *et al.*, 2020). Factors influencing the EO composition include extraction methods, plant part and genotype, geographical origin, environmental conditions, harvest period, drying processes and conditions (Paolini *et al.*, 2010). This study demonstrated that blended EOs exhibited higher antibacterial activity than single oils, with significantly larger inhibition zones ($p<0.05$). The enhanced effect of the blended EO may be due to the synergistic interaction of major components, which collectively influence bacterial physiological pathways. For example, PAGE8-epinenone-4-ol, the principal element of tea tree oil, increases bacterial membrane permeability (Carson *et al.*, 2002), while cinnamaldehyde and eugenol from cinnamon oil are known antibacterial agents (Oussalah *et al.*, 2006). Additionally, thymol exhibits antitumor effects through mechanisms such as cell growth inhibition and apoptosis induction (Kowalczyk *et al.*, 2020) and carvacrol and thymol disrupt bacterial membranes, prevent microbial mobility, and block bacterial efflux pumps (Rathod *et al.*, 2021; Rota *et al.*, 2007; Ultee *et al.*, 2000). Blended oils, such as a combination of clove EO and vanillin/cinnamon bark EO, have shown synergistic antimicrobial effects compared to single oils (Cava-Roda *et al.*, 2021). MIC values serve as an important measure for quantifying the antimicrobial movement of essential oils and evaluating their potential for clinical claims. In particular, bergamot, peppermint, rosemary, and orange oils are stated to have low MIC values against pathogens, showing their potential as natural antimicrobial agents. This synergy is reflected in the reduced MIC values, where the blended oils lower the MIC by 2–6 times compared to individual oils (Pinto *et al.*, 2023). This decrease suggests that the compounds within the blends interact synergistically to boost the antimicrobial effect, enabling effective action at lower concentrations.

With the growing concern about synthetic antimicrobial agents and the increasing prevalence of multidrug-resistant bacteria, EOs present a promising alternative therapeutic strategy (Pieri *et al.*, 2020). Earlier research has revealed that EOs exhibit potent antibacterial effects similar to antibiotics such as gentamicin, streptomycin, and colistin against *E. coli* (Zouari *et al.*, 2010). Moreover, EOs have demonstrated superior anti-MRSA activity, outperforming conventional antibiotics such as ciprofloxacin and oxacillin (Shamsudin *et al.*, 2022). This study indicates that combining EOs can significantly enhance antibacterial activity, especially in S. aureus, MRSA, E. coli, and P. aeruginosa. These results offer new insights into the complex mechanisms behind EO effectiveness and support the development of natural antibacterial agents. In the future, research should concentrate on assessing the antimicrobial efficacy of blended EOs in a broader variety of pathogens, with multidrug-resistant bacteria, fungi, and illnesses, by quantifying synergy using established models like the Fractional Inhibition Concentration Index (FICI), which would further expand the therapeutic potential of EOs. Furthermore, future clinical or in vivo validation is essential to confirm its clinical applicability and determine optimal concentrations for specific applications such as skin infections, wound healing, and oral hygiene and to explore using EO in medical and private care products. Addressing the challenges of EO solubility and volatility through formulation studies can further enhance its academic, industrial, and clinical applications as a natural antimicrobial agent.

Conclusion

This research highlights a promising antibacterial possible of blended EOs against key pathogens like *S. aureus*, MRSA, *E. coli*, and *P. aeruginosa*.

The findings highlight the superior efficacy of blended EOs over individual oils, implying that the synergistic interactions between the active components contribute to enhanced antibacterial activity. While gram-positive bacteria were more liable to EOs than gram-negative bacteria, findings emphasize a need for further investigation into EO formulations to combat multidrug-resistant pathogens. The rising threat of antibiotic resistance makes essential oils an appealing alternative therapeutic approach. Future studies should aim to broaden the range of pathogens investigated, identify optimal doses for particular applications, and explore the clinical use of EOs in treating infections, especially those caused by resistant bacteria. Furthermore, addressing the issues of EO solubility and

volatility through improved formulations has the potential to boost their effectiveness as natural antibacterial agents in both medicinal and consumer applications.

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Author's Contributions

Min-Gi Kwon: Wrote the manuscript and data analysis.

Jae Kyung Kim: Prepare original draft, write review, and edit.

Ethics

Ethical approval was not required for this study.

Conflicts of Interest

This study was conducted without any external funding.

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