

In vitro antibacterial activity of ethanolic peel and seed extracts of sweet orange (*Citrus sinensis*) against *Staphylococcus aureus* and *Escherichia coli*

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Abstract

Background

Antimicrobial resistance in common pathogens such as *Staphylococcus aureus* and *Escherichia coli* has renewed interest in antibacterial agents derived from plants. The peels and seeds of sweet orange (*Citrus sinensis*) are abundant agro-processing residues that are rich in bioactive compounds but are usually discarded. This study compared the in vitro antibacterial activity of ethanolic peel and seed extracts of *C. sinensis* against a Gram-positive and a Gram-negative organism.

Methods

Dried peels and seeds were extracted by ethanolic maceration, and the crude extracts were reconstituted in 2% dimethyl sulfoxide to give concentrations of 20, 40, 60 and 80% w/v (200–800 mg/mL). Antibacterial activity against *S. aureus* (ATCC 43300) and *E. coli* (ATCC 25922) was determined in triplicate by the agar well diffusion method, with gentamicin and sterile distilled water as positive and negative controls. Zones of inhibition were analysed by one-way and two-way analysis of variance (ANOVA) and independent-samples t-tests.

Results

Both extracts inhibited the two organisms in a concentration-dependent manner. The largest zones were produced by the 80% peel extract against *S. aureus* (12.33 ± 0.58 mm) and *E. coli* (12.33 ± 1.53 mm). One-way ANOVA confirmed a significant effect of concentration for every extract–organism combination ($p < 0.05$). Two-way ANOVA identified concentration as the dominant factor for both organisms, with a significant extract $\times$ concentration interaction for *E. coli* ($p = 0.046$) but not for *S. aureus* ($p = 0.705$). The peel produced numerically larger zones than the seed, but the difference was not statistically significant (*S. aureus*, $p = 0.091$, Cohen's $d = 0.85$; *E. coli*, $p = 0.840$). *S. aureus* was generally more susceptible than *E. coli*.

Conclusions

Ethanolic peel and seed extracts of *C. sinensis*, and particularly the peel, showed concentration-dependent antibacterial activity against both test organisms. The findings support the potential of sweet orange processing residues as a low-cost source of antibacterial compounds and add value to a commonly discarded agro-waste, while indicating the need for determination of minimum inhibitory and bactericidal concentrations and for phytochemical characterisation in future work.

Background

Bacterial infections remain a heavy burden on human and animal health, and the steady rise of antibiotic-resistant strains of *Staphylococcus aureus* and *Escherichia coli* has made their treatment increasingly difficult (Amos, 2025). These two organisms are responsible for conditions ranging from minor skin and wound infections to life-threatening systemic disease (Abalaka & Bello, 2016). The problem is compounded by the adverse effects of some conventional antibiotics and by the way their overuse selects for resistance, which together narrow the range of agents that remain reliably effective (Dubey et al., 2011). Against this background, there is growing interest in complementary antibacterial agents that are affordable and locally available (Ashritha et al., 2021), and medicinal plants used for this purpose long before the antibiotic era are an obvious place to look (Musa et al., 2019).

Sweet orange (*Citrus sinensis*) is one of the most widely grown and consumed fruits in the world (Afroja et al., 2017). Its peels and seeds, which accumulate in large quantities as juice- and processing-waste, contain flavonoids, phenolic acids, tannins, alkaloids and essential oils, several of which have documented antimicrobial activity (Dang-Bao et al., 2022). Recovering these residues for use as antibacterial agents therefore makes both environmental and economic sense, particularly in settings where fruit waste is abundant (Ramadan et al., 2015). The compounds concerned are thought to act by disrupting bacterial cell walls and membranes, inhibiting key enzymes, and interfering with nucleic-acid synthesis (Shehata et al., 2021).

Previous studies have shown that *C. sinensis* extracts inhibit a range of bacteria, that peel extracts are generally more active than seed extracts, and that ethanolic extraction tends to recover more of the active constituents than water; however, the reported zone sizes vary widely with fruit variety, maturity and extraction method (Egbuonu & Osuji, 2016). Much of this work has focused on the peel or on the juice (Hassan et al., 2021; Iwuji et al., 2022), and comparatively few studies place peel and seed side by side within a single experiment (Egbuonu & Amadi, 2016). Fewer still test whether extract type and concentration interact that is, whether any advantage of the peel is uniform across organisms or depends on the bacterium and the dose. The present study was designed to address these gaps. Working with two reference strains of contrasting cell-wall architecture, we compared the antibacterial activity of ethanolic peel and seed extracts of *C. sinensis* across a range of concentrations, and used a factorial analysis to separate the effects of extract type, concentration and their interaction.

Methods

Collection and preparation of plant material

Fresh, healthy sweet oranges (*C. sinensis*), free from visible damage or decay, were purchased from a local market in Morogoro, Tanzania. The fruits were washed under running tap water and rinsed with distilled water. The peels and seeds were separated using sterile instruments, dried in an oven at a temperature not exceeding 40°C for three days to constant weight so as to preserve the bioactive constituents (Ramadan et al., 2015), and then milled separately into fine powders using a clean electric blender.

Preparation of extracts

Peel powder (189.1 g) and seed powder (41.5 g) were each macerated in 100 mL of 95% ethanol (solvent-to-material ratios of approximately 0.53:1 mL/g for the peel and 2.41:1 mL/g for the seed) for 72 h at room temperature with intermittent shaking. Each mixture was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated to dryness under reduced pressure in a rotary evaporator to yield the crude extract, which was stored at 4°C until use. Working concentrations of 20, 40, 60 and 80% w/v were prepared by dissolving 0.2, 0.4, 0.6 and 0.8 g of crude extract, respectively, in 1 mL of 2% dimethyl sulphoxide (DMSO); these correspond to 200, 400, 600 and 800 mg/mL. Ethanol extraction was chosen because it recovers phenolic and flavonoid constituents more efficiently than aqueous extraction (Oikeh et al., 2020).

Test organisms and inoculum

Reference strains of *S. aureus* (ATCC 43300) and *E. coli* (ATCC 25922) were obtained from a recognised culture collection and maintained on nutrient agar, with *E. coli* also checked on MacConkey agar. Before testing, each organism was sub-cultured to obtain an active culture, and the inoculum was standardised in sterile saline to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL).

Agar well diffusion assay

Antibacterial activity was determined by the agar well diffusion method in triplicate. Mueller-Hinton agar was prepared according to the manufacturer's instructions, sterilised by autoclaving at 121°C for 15 min, poured into sterile Petri dishes and allowed to set. The standardised inoculum of each organism was spread evenly over the agar surface, and wells of 6 mm diameter were bored using a sterile cork borer. A fixed volume (0.2 mL) of each extract concentration was dispensed into the appropriate wells.

Gentamicin served as the positive control and sterile distilled water containing an equal volume of the vehicle served as the negative control. Plates were incubated at 37°C for 24 h, after which the diameter of the zone of inhibition around each well was measured in millimetres with a calibrated ruler. One seed replicate at the 60% concentration was excluded from analysis because the plate was compromised.

Statistical analysis

Zones of inhibition are reported as mean $\pm$ standard deviation of triplicate measurements. The effect of concentration within each extract-organism combination was tested by one-way ANOVA. A two-way ANOVA, balanced at the 20, 40 and 80% levels, was used to test the effects of extract type, concentration and their interaction. Peel and seed extracts were compared directly for each organism by independent-samples t-test, with Cohen's d as a measure of effect size. Differences were considered significant at $p < 0.05$. Analyses were performed in Python (SciPy and statsmodels).

Results

Antibacterial activity and concentration dependence

Both extracts produced clear zones of inhibition against the two organisms, and the zones widened as the concentration increased (Tables 1 and 2). The negative control produced no inhibition, confirming that activity was due to the extracts rather than the vehicle. The 80% peel extract gave the largest zones against both *S. aureus* (12.33 ± 0.58 mm) and *E. coli* (12.33 ± 1.53 mm). Gentamicin produced a larger zone against *E. coli* (20.00 mm) than against *S. aureus* (10.00 mm); against *S. aureus* the 80% peel extract slightly exceeded the gentamicin zone, a result most likely reflecting the antibiotic loading of the control and the susceptibility of the particular reference strain rather than a true superiority of the crude extract over the antibiotic.

Table 1
Zone of inhibition (mm) of *Citrus sinensis* peel and seed extracts against *Staphylococcus aureus*

Extract	Conc. (%)	Rep 1	Rep 2	Rep 3	Mean	SD
Peel	20	10	7	7	8.00	1.73
Peel	40	12	9	8	9.67	2.08
Peel	60	12	10	11	11.00	1.00
Peel	80	13	12	12	12.33	0.58
Seed	20	6	7	7	6.67	0.58
Seed	40	9	8	8	8.33	0.58
Seed	60	NA	NA	NA	NA	NA
Seed	80	10	10	10	10.00	0.00
Gentamicin	-	11	10	9	10.00	1.00
Distilled water	-	0	0	0	0.00	0.00

NA, not available (compromised plate excluded from analysis).

Table 2
Zone of inhibition (mm) of *Citrus sinensis* peel and seed extracts against *Escherichia coli*

Extract	Conc. (%)	Rep 1	Rep 2	Rep 3	Mean	SD
Peel	20	6	7	7	6.67	0.58
Peel	40	6	9	8	7.67	1.53
Peel	60	11	10	10	10.33	0.58
Peel	80	14	12	11	12.33	1.53
Seed	20	6	7	8	7.00	1.00
Seed	40	8	10	9	9.00	1.00
Seed	60	NA	NA	NA	NA	NA
Seed	80	9	11	10	10.00	1.00
Gentamicin	-	21	20	19	20.00	1.00
Distilled water	-	0	0	0	0.00	0.00

NA, not available (compromised plate excluded from analysis).

One-way ANOVA confirmed that concentration significantly affected activity for every extract–organism combination (Table 3). The effect was significant for the peel against *S. aureus* and highly significant for the seed against *S. aureus* and for the peel against *E. coli*.

Table 3
One-way ANOVA of the effect of concentration on zone of inhibition

Bacterium	Extract	F (df)	p-value	Significance
<i>S. aureus</i>	Peel	F(3,8) = 4.76	0.035	Significant
<i>S. aureus</i>	Seed	F(2,6) = 37.50	< 0.001	Highly significant
<i>E. coli</i>	Peel	F(3,8) = 14.90	0.001	Highly significant
<i>E. coli</i>	Seed	F(2,6) = 7.00	0.027	Significant

df, degrees of freedom.

Effect of extract type, concentration and their interaction

The two-way ANOVA (Table 4) identified concentration as the dominant, highly significant factor for both organisms. Extract type had a significant effect for *S. aureus* but not for *E. coli*, and a significant extract ×

concentration interaction was present for *E. coli* only. This indicates that the relative performance of peel and seed was not uniform, but depended on both the organism and the dose.

Table 4
Two-way ANOVA of extract type × concentration for each organism

Source	df	F	p-value	Sig.
<i>S. aureus</i> - Extract type	1	9.00	0.011	*
<i>S. aureus</i> - Concentration	2	15.96	< 0.001	***
<i>S. aureus</i> - Extract × conc.	2	0.36	0.705	ns
<i>E. coli</i> - Extract type	1	0.17	0.690	ns
<i>E. coli</i> - Concentration	2	21.79	< 0.001	***
<i>E. coli</i> - Extract × conc.	2	4.04	0.046	*

* $p < 0.05$; *** $p < 0.001$; ns, not significant; df, degrees of freedom.

Direct comparison of peel and seed extracts

Although the peel produced numerically larger mean zones than the seed, the independent-samples t-test found no significant difference for either organism (Table 5). For *S. aureus* the effect size was nonetheless large (Cohen's $d = 0.85$), suggesting a real difference that the small sample was underpowered to detect; for *E. coli* the effect was negligible.

Table 5
Independent-samples t-test comparing peel and seed extracts (concentrations 20, 40 and 80%)

Bacterium	t (df)	p-value	Cohen's d
<i>S. aureus</i>	$t(16) = 1.80$	0.091	0.85 (large)
<i>E. coli</i>	$t(16) = 0.20$	0.840	0.10 (negligible)

df, degrees of freedom.

Graphical summary

The concentration-dependent activity of the two extracts against each organism is shown in Fig. 1, the direct comparison between organisms in Fig. 2, and the dose–response relationship on a mass basis in Fig. 3.

Bars represent mean $\pm$ SD of triplicate measurements; the 60% seed concentration was not read for one replicate and is excluded.

Bars represent mean $\pm$ SD of triplicate measurements.

Discussion

Both the peel and the seed extracts of *C. sinensis* inhibited *S. aureus* and *E. coli*, and in every case the zones of inhibition widened as the extract concentration rose. The one-way ANOVA confirmed that this concentration effect was significant throughout, and the two-way ANOVA showed it to be the dominant factor overall. A dose-dependent response of this kind is consistent with the idea that higher concentrations deliver more of the active phytochemicals to the bacterial cells (Oikeh et al., 2020), and it agrees with earlier reports for citrus extracts (Keerthana & Ramesh, 2021).

The peel extract produced numerically larger zones than the seed extract against both organisms, in line with the widely held view that the peel is richer in essential oils and phenolic compounds than the seed (Egbuonu & Osuji, 2016; Olabanji et al., 2016). It is worth being cautious here, however. The direct t-test found no significant peel-seed difference for either organism, even though the effect size for *S. aureus* was large ($d = 0.85$). The most likely explanation is limited statistical power: with only three replicates per level, a genuine difference of this magnitude can easily fail to reach significance. The two-way ANOVA adds a further nuance, in that the extract $\times$ concentration interaction was significant for *E. coli* but not for *S. aureus*. Taken together, these results suggest that the peel's apparent advantage is real but modest, and that it is not uniform across organisms a more measured conclusion than a simple ranking of peel over seed would imply.

S. aureus was generally more susceptible to both extracts than *E. coli*. This is consistent with the difference in cell-envelope architecture between the two organisms. *S. aureus* is Gram-positive, with a thick but relatively permeable peptidoglycan wall and no outer membrane, so antibacterial compounds can reach their targets fairly easily. *E. coli* is Gram-negative and carries an additional outer membrane of lipopolysaccharide that acts as a permeability barrier, and Gram-negative bacteria are for this reason often less susceptible to plant-derived antibacterials (Shehata et al., 2021). The activity we observed is attributable to the phytochemicals characteristic of citrus by-products such as flavonoids, phenolic acids, tannins, alkaloids and essential oils in which d-limonene predominates—which act on the bacterial membrane and on intracellular targets (Dang-Bao et al., 2022; Haroen et al., 2018).

The zone diameters recorded here (roughly 7–12 mm) fall within the broad range reported for crude *C. sinensis* extracts, although absolute values differ between studies because of variation in fruit variety, solvent and assay conditions (Afroja et al., 2017; Jamilu & Lema, 2024). The comparatively modest zones seen at the lowest concentrations, some only slightly larger than the 6 mm well, indicate weak activity at those doses and reinforce the importance of concentration. The wide zone produced by gentamicin against *E. coli*, contrasted with its narrower zone against this particular *S. aureus* strain, is a reminder that reference-antibiotic behaviour is strain- and loading-dependent and should be interpreted with care.

From an applied standpoint, the results support the view that orange peel discarded in large amounts as processing waste can serve as an inexpensive source of antibacterial compounds, with possible value in pharmaceutical, food-preservation and public-health contexts, and with the added benefit of reducing fruit-waste (Musa et al., 2019; Ramadan et al., 2015). Realising that potential will require work beyond the scope of the present study.

Several limitations should be acknowledged. Only two reference organisms were tested, using a single method (agar well diffusion), and the individual phytochemicals responsible for activity were neither isolated nor quantified. The small number of replicates limited statistical power, and one seed data point was lost to a compromised plate. In addition, the peel and seed powders were macerated in a fixed ethanol volume (100 mL) rather than a standardised solvent-to-material ratio, giving a lower ratio for the peel (approximately 0.53:1 mL/g) than for the seed (approximately 2.41:1 mL/g); if anything, this would be expected to favour extraction efficiency for the seed rather than the peel, so it is unlikely to explain the peel's apparent advantage, but standardising the ratio would strengthen future comparisons. Future work should determine minimum inhibitory and minimum bactericidal concentrations, characterise the active constituents for example by quantifying total phenolics and flavonoids and by profiling the peel oil test a wider panel of pathogens including field and drug-resistant isolates, and assess the safety of the extracts before any applied use is considered.

Conclusions

Ethanol peel and seed extracts of *C. sinensis* showed concentration-dependent antibacterial activity against *S. aureus* and *E. coli*, with the peel tending to outperform the seed and with *S. aureus* the more susceptible of the two organisms. The peel–seed difference, although supported by a large effect size for *S. aureus*, did not reach significance and was not uniform across organisms, so it should be regarded as promising rather than established. The findings nonetheless indicate that sweet orange processing residues are a low-cost, accessible source of antibacterial compounds and a candidate for agro-waste valorisation, and they justify further work on minimum inhibitory and bactericidal concentrations and on the identity of the active constituents.

Abbreviations

ANOVA

Analysis of variance

ATCC

American Type Culture Collection

CFU

Colony-forming unit

df

Degrees of freedom

DMSO

Dimethyl sulphoxide

MBC

Minimum bactericidal concentration

MIC

Minimum inhibitory concentration

SD

Standard deviation

w/v

Weight per volume

ZOI

Zone of inhibition

Declarations

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Authors' contributions

GRG conceived and carried out the laboratory investigation, curated the data and drafted the manuscript. RRS conceived and supervised the study, contributed to the methodology and the analysis, PSB, VEM, DJJ, MAM; contributed to manuscript writing and reviewed and edited the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials

All data generated and analysed during this study are included in this article. The raw datasets are also available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable. This study did not involve human participants or animals; it used reference bacterial strains only.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Antibacterial activity of *Citrus sinensis* peel and seed extracts

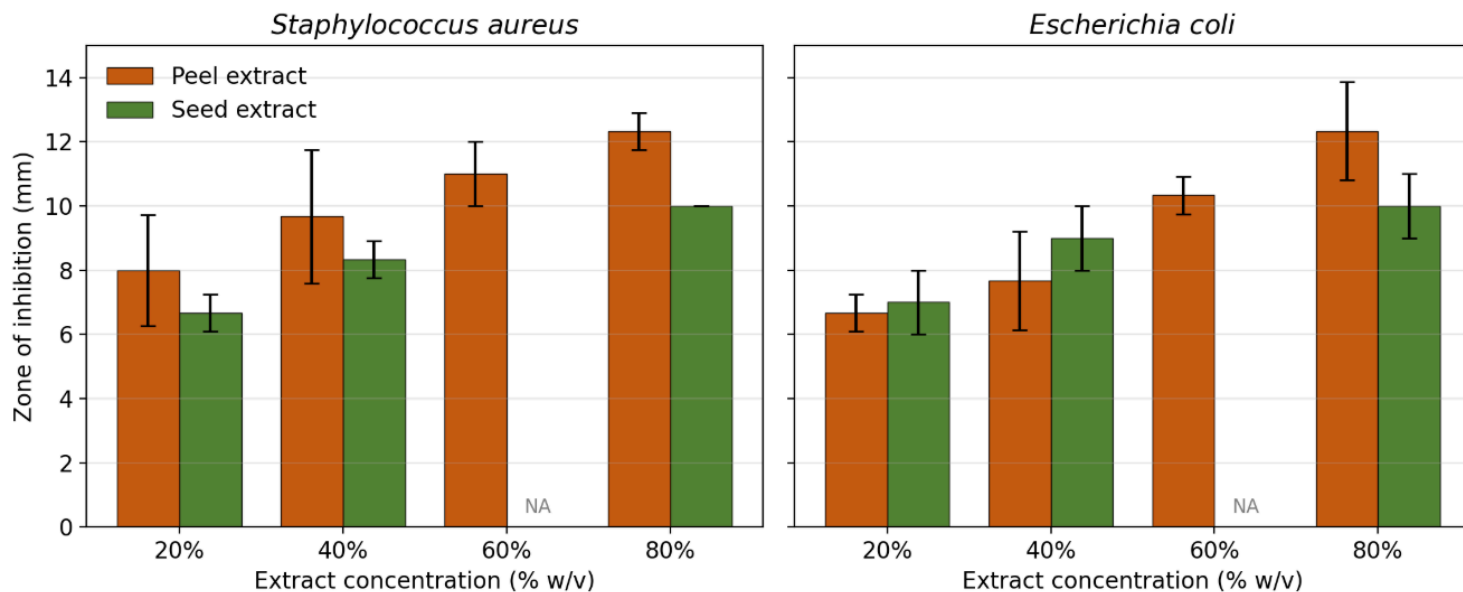


Figure 1

Antibacterial activity of *Citrus sinensis* peel and seed extracts against *S. aureus* and *E. coli*.

Bars represent mean $\pm$ SD of triplicate measurements; the 60% seed concentration was not read for one replicate and is excluded.

Comparison of antibacterial efficacy against the two test organisms

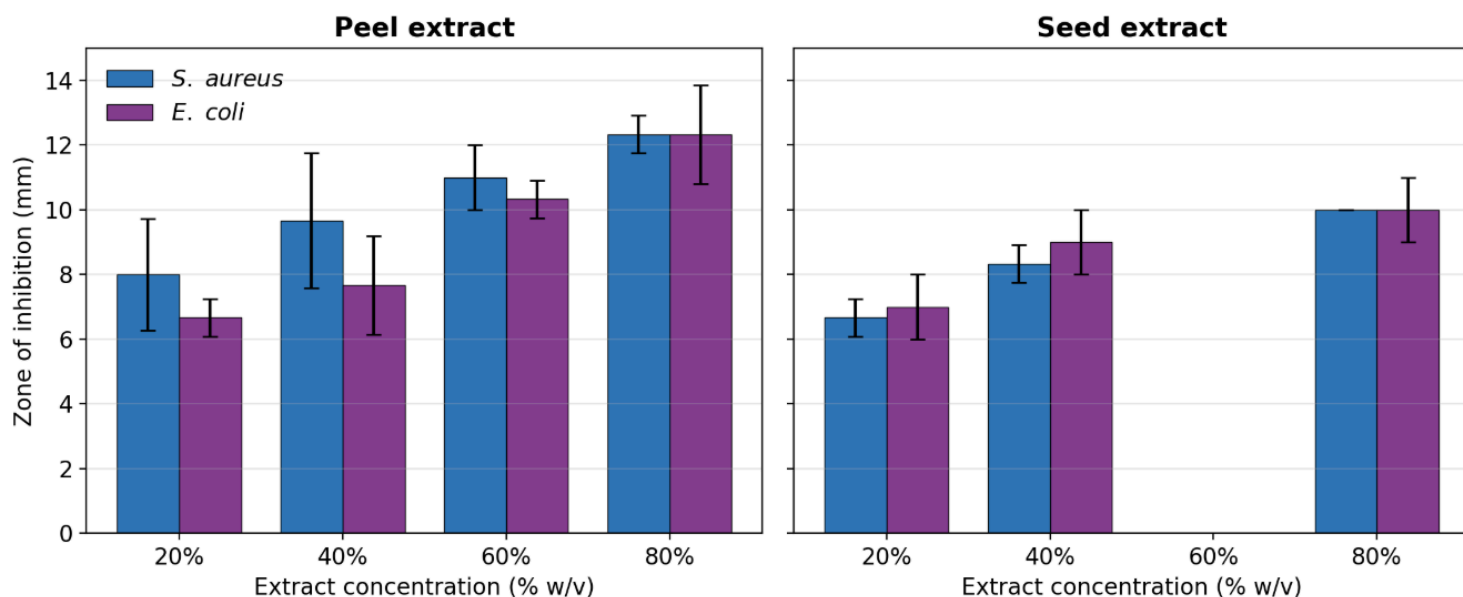


Figure 2

Comparison of antibacterial efficacy of peel and seed extracts against the test organisms.

Bars represent mean $\pm$ SD of triplicate measurements.

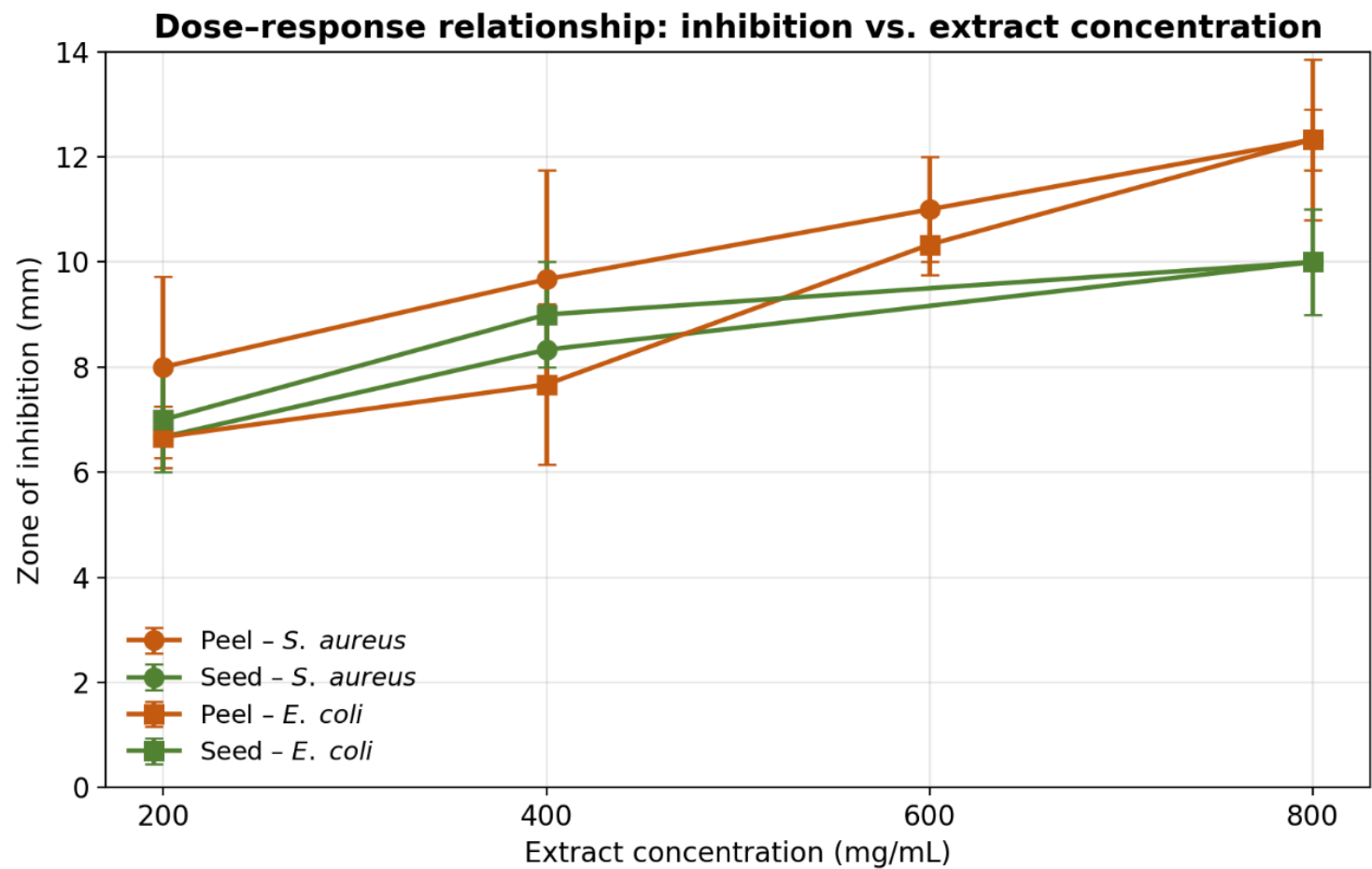


Figure 3

Dose-response relationship between extract concentration (mg/mL) and zone of inhibition