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EVALUATION OF WALNUT GREEN HUSK, *CAPPARIS SPINOSA*, *CISTANCHES HERBA*, *ECLIPTA PROSTRATA*, *SCUTELLARIA BARBATA*, *SARGENTODOXA CUNEATA*, AND *SOPHORA FLAVESCENS* EXTRACTS AGAINST *STAPHYLOCOCCUS AUREUS*, *ESCHERICHIA COLI*, AND *SALMONELLA ENTERITIDIS*

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ABSTRACT

Herbs are widely used in the treatment of a wide range of illnesses in humans and animals. They contain an array of natural antibacterial compounds and possess potent antibacterial properties, making them a promising source of low-toxicity, high-efficiency alternative antimicrobial agents that are less likely to develop drug resistance. In this experiment, total phenols and flavonoids, free radical scavenging ability of extracts derived from seven traditional Chinese herbs, including walnut (*Juglans regia*) green husk, *Cistanche herba*, *Capparis spinosa*, *Eclipta prostrata*, *Sophora flavescens*, *Scutellaria barbata*, and *Sargentodoxa cuneata*, were investigated. Furthermore, the antibacterial properties of the extracts against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enteritidis* were assessed. The results showed that the concentrations of total phenols and flavonoids of the seven extracts ranged from 5.45 to 226.89 mg gallic acid equivalent (GAE)/mL and 7.34 to 428.04 mg rutin equivalent (RE)/mL, respectively. *S. cuneata* extract exhibited the highest contents of total phenols and flavonoids, which were significantly higher than those of the other herbs extract ($P < 0.05$). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging rates of the extracts were ranged from 28.25% to 85.33%. The extracts from *S. cuneata* and *E. prostrata* showed the highest antioxidant activity as compared to other extracts ($P < 0.05$). Extract from the walnut green husk possessed the best antibacterial properties against *S. aureus* and *S. enteritidis* with inhibition zone diameters of 13.05 and 8.59 mm, respectively, which were significantly greater than those of the other herbal extracts ($P < 0.05$). For *E. coli*, *S. barbata* extract displayed the highest antibacterial activity of with a diameter of inhibition zone of 9.83 mm. Based on the findings of the current study, it is concluded that the extracts from *S. cuneata* and *E. prostrata* exhibited favorable antioxidant capacity and the extracts derived from the walnut green husk and *S. barbata* have the potential to serve as alternatives for antibiotics.

Keywords: Herbs, phenols, flavonoids, antioxidant activity, antibacterial properties

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INTRODUCTION

Bacterial antimicrobial resistance refers to the insensitivity of bacteria to antibiotic treatment, which is one of the leading public health threats of the 21st century (Gordon *et al*., 2005). A recent study has reported that in less than 30 years, antimicrobial resistance may be more deadly than cancer and will kill 10 million people a year by 2050 (Antimicrobial Resistance Collaborators, 2022). It could also be speculated that increase in bacterial drug resistance may cause server challenges to prevent and treat drug-resistant bacterial infections. Although antibiotics are being used to treat most of the bacterial infections, however, the emergence of antimicrobial resistance weakening the effectiveness of existing antibiotics. Therefore, it is important to find effective alternatives of antibiotic with favorable antimicrobial activity both for humans and animals. At present, herbs have become a promising antimicrobial strategy because herbs contain a diverse range of active components, including flavonoids, alkaloids, phenols, and quinones, which exhibit quality antibacterial properties as presented in recent studies (Li *et al*., 2022a; Metin and Bürü, 2023).

Various kind of plants including herbs have been used as medicines for more than 5000 years (Brown and Wright, 2016), as a source of antibiotics, antineoplastic, analgesics, and cardioprotective (Chen *et al*., 2015). It has been reported that about 70–90% of the population in developing countries are using ancient

medicines based on plant extracts (Chin *et al*., 2006). Interestingly, it has also been reported that natural products and their derivatives contribute to more than half of the Food and Drug Administration approved drugs (Chavan *et al*., 2018). In China, interest on use of walnut (*Juglans regia*) green husk, *Capparis spinosa*, *Cistanches herba*, *Eclipta prostrata*, *Scutellaria barbata*, *Sargentodoxa cuneata*, and *Sophora flavescens* as traditional herbal medicines has developed due to their known effect as anti-tumor, anti-aging, and anti-inflammatory. However, very few studies on walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* effect as antibacterial are available (Zeb, 2018; Li *et al*., 2022b). For example, more than 240 phytochemicals have been identified from walnut green husk, including quinones, flavonoids, ellagic acid, and tannins, which exhibit anti-tumor, and immune-regulating activities and being used to treat cancers, tumors, dysentery, diarrhea and oxidative stress (Zeb, 2018; Li *et al*., 2022b). The *C. herba* is a plant with dual purpose in medicine and food and known for its nutritional benefits in improving kidney and heart functions, and gut health. The *C. herba* exhibits pharmacological effects such as anti-tumor, anti-aging, and immune-enhancing properties and glycosides, cynarin, and echinacoside compound in *C. herba* play a positive role in the treatment of Alzheimer's disease (Gu *et al*., 2016; Zhou *et al*., 2023). The *C. spinosa* is another herb and its medicinal purposes was first documented in Chinese "Yao Dian" (Pharmacopeia) in the 11th-century (Kdimy *et al*., 2022). It has been reported that extract derived from *C. spinosa* contains polyphenols and other active compounds and have therapeutic properties, like anti-diabetic and hypolipidemic effects, and organ protection (Vahid *et al.*, 2017). Similar with *C. spinosa, E. prostrata* is an herb that contains a variety of compounds, including steroids, flavonoids, and thiophene derivatives and *E. prostrata* is effective in treating allergic and inflammatory diseases (Kang *et al*., 2022). *S. flavescens* is another important medicinal plant known with wide range of pharmacological effects due including anti-tumor, antibacterial, antipyretic, analgesic, and anti-inflammatory effects (He *et al.*, 2015) and it is being used to prevent and treat chronic diseases such as cardiovascular disease and tumors (Sun *et al.*, 2022a). Another famous herb is *S. barbata* that contains triterpenoids, polysaccharides, and essential oils and being used to treat tumor diseases (Wang *et al*., 2019). *S. cuneata* is also an endemic plant of the *Sargentodoxae* family in China, and it contains numerous chemical components that exhibit significant anti-inflammatory effects (Guo *et al.*, 2018). In summary, walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* are potential traditional herbal medicines that are being used against various diseases and have nutritional benefits and anti-tumor, anti-aging, and anti-inflammatory, anticancer effects, however, data of their use as antibacterial is limited. Therefore, the objective of the current study was to evaluate the effect of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enteritidis.*

MATERIALS AND METHODS

Herbs, strains and reagents: Naturally air-dried walnut green husk, *C. herba*, *C. spinosa*, *E. prostrata*, *S. flavescens*, *S. barbata*, and *S. cuneata* were purchased from the Xinjiang Baicaotang Pharmacy (Urumqi, China). All herbs were crushed with a grinder and passed through a 0.15 mm sieve, and the powders were stored in sealed bags and preserved at -20℃ for future use. *S. aureus* (CMCC 26003), *E. coli* (CMCC 8739), and *S. enteritidis* (CMCC 50041) were gifted by Professor Xiaowei Bao of Xinjiang Agricultural University. Flavomycin with a purity of 99% was obtained from North China Pharmaceutical Group (Hebei, China). The culture media were purchased from Sangong Bioengineering Co, Ltd (Shanghai, China). All of other reagents utilized were of analytical grade.

Preparation of herbal extracts: Previous study has reported that 50% hydroalcoholic solution effectively extract bioactive compound (anthocyanins) from wine lees, jabuticaba by-products, and eggplant peels (Ferarsa *et al*., 2018; Romero-Díez *et al*., 2019; Tarone *et al*., 2021). While, another study reported that 70% hydroalcoholic solution allows the best yield extraction of bioactive compound (Gómez-García and Ochoa-Alejo, 2013). However, a recent study reported that 90% hydroalcoholic solution not only result in higher extraction of bioactive compounds from ginseng but its bioactive compounds showed strongest antioxidant activity (Huang *et al*., 2019). In addition, among the emerging technologies, ultrasonic extraction is one of the most explored and effective technology to recover bioactive compounds from plants, herbs and by-products (Wani *et al*., 2021; Sharma *et al*., 2021) and studies of Tiwari (2015) and Chemat *et al*. (2017) reported that ultrasonic extraction not only decrease extraction and processing time, energy, carbon monoxide emissions and solvents use but also enhance extraction yield and enhance extraction of heat-sensitive components during extraction process. Therefore, in the current study, 90% hydroalcoholic solution coupled with ultrasonic extraction was carried out not only for maximum extraction yield but also for safe yield of bioactive compound in the extract. In brief, the weighed herbal powders from all seven sources were mixed with 90% hydroalcoholic solution at a ratio of 1:20 (w/v) in a 500 mL flask. The extraction process was conducted for 2 h at

55℃ using an ultrasonic extractor (KQ-5200, Kunshan Ultrasonic Instrument Co., Ltd, Jiangsu, China) with an ultrasonic power of 200W. After standing for 12 h, the residue was filtered with an 8-layer gauze cloth. Subsequently, the filtrates were concentrated using a rotary evaporator (R-3001, The Greatwall Scientific Industrial and Trade Co., Ltd, Zhengzhou, China) at 55℃. The concentrated extracts were then completely dried at 55℃. The resulting extracts were then used for subsequent assays to evaluate their antimicrobial activity.

Total phenols: The dried extracts from walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* were dissolved in 90% ethanol solution to achieve a final concentration of 200 mg/mL. Total phenolic content was measured using the Folin-Ciocalteu method (Lopez-Froilan *et al*., 2018; Meng *et al*., 2020), which, while widely used, may overestimate phenolic content due to interference from other reducing substances. Nevertheless, some authors have studied the use of different methods to clean up the interference substances and alternative Folin-Ciocalteu reacting conditions to limit total phenolic overestimation (Georgé *et al*., 2005; Carmona-Hernandez *et al*., 2021). Therefore, total phenolic contents of the extracts in the current study were determined as descripted by Meng *et al*., (2020) with some modifications of previously described method (Lopez-Froilan *et al*., 2018). In brief, 1.0 mL of diluted extract solution of each herb mixed with 1.0 mL of Folin-Ciocalteu phenol reagent, and the resulting mixture was added with 3.0 mL of 12.5% sodium carbonate (Na_2CO_3) solution and 5.0 mL of double-distilled water (ddH_2O). The mixture was incubated for 1 h at 30 °C in a water bath. To determine the total phenolic content in the extracts, gallic acid was employed as a reference, and absorbance was measured with a spectrophotometer (SPC-752, Shanghai Spectrum Instruments Co., China) at 764 nm. The total phenolic content in the extracts was quantified and reported as mg/mL gallic acid equivalent (mg GAE/mL).

Total flavonoids: The total flavonoids were determined by using aluminum chloride colorimetric method (Kalia *et al*., 2008) with some modification as described previously (Chen *et al*., 2020) because that method was specific to flavonoids but may not capture all types of flavonoid compounds present in the extracts. In brief, a volume of 1.0 mL of each extract was added with an 80% ethanol solution to give a total volume of 10.0 mL. Next, 1.0 mL of a 5% sodium nitrite ($NaNO_2$) solution was added and mixed thoroughly. After resting for 6 min, 1.0 mL of 10% aluminum nitrate ($Al(NO_3)_3$) solution was supplemented and well mixed. The mixture was allowed to settle for another 6 min before 10.0 mL of a 10% sodium hydroxide (NaOH) solution was added. Finally, ddH_2O was added to achieve a final volume of 25.0 mL. After shaking and standing for 15 min, the absorbance value was assayed at 510 nm. The total flavonoid contents were quantified using rutin as a reference and reported as mg/mL rutin equivalent (mg RE/mL).

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging capacity: The DPPH free radical scavenging rates of the extracts were determined as in a previous report (Hussen *et al.*, 2023). Briefly, 0.3 mL of the extracts was added with 2.7 mL of 0.1 mol/L DPPH anhydrous ethanol solution and well mixed. After incubation in the dark for 0.5 h, the absorbance value at 517 nm (OD_{sample}) was recorded. The absorbance value of the mixture of 0.3 mL of the extracts and 2.7 mL of anhydrous ethanol was measured and recorded as OD_{blank}. Similarly, the absorbance value of the mixture of 0.3 mL of 90% ethanol and 2.7 mL of DPPH solution was measured and labeled as OD_{DPPH}.

$$\text{DPPH free radical scavenging rate (\%)} = \frac{OD_{DPPH} - (OD_{sample} - OD_{blank})}{OD_{DPPH}} \times 100\%$$

Inhibition zone diameters (IZD): The IZDs of the extracts against *S. aureus*, *S. enteritidis*, and *E. coli* were determined as descripted by Zou *et al*. (2022). In brief, inoculum was standardized (bacterial inoculum was prepared using a standardized protocol to achieve a consistent concentration of 1.0×10^8 colony-forming units (CFU)/mL), and a volume of 0.10 mL standardized inoculum was mixed with 15.0 mL of Luria–Bertani (LB) agar medium at 60 ℃ and poured into petri dish. LB agar medium used in this method was prepared in large batches and poured into petri dishes to ensure uniform thickness and consistency across all plates. After the medium solidified, Oxford cups (Φ8.0 × 6 × 10 mm) were evenly placed on the medium (create wells in the agar), and 0.2 mL of the extract at a concentration of 200 mg/mL was added into each cup. After diffusion for 12 h at 4 °C, the bacteria were cultured in an incubator (DH-360AS, Kewei Yongxing Instrument Co., Beijing, China) at 37°C for 24 h. Each treatment was performed in triplicate, and both positive (50 mg/mL of flavomycin in 90% ethanol) and negative (90% ethanol) controls were included in each batch of assays. IZD was measured by the cross method and subtracted by 8 mm (diameter of the Oxford cup).

Minimum Inhibitory Concentration (MIC): The MICs of the extracts were assayed according to the method reported by Zou *et al*. (2022) with slight modifications. The extracts were diluted 2-fold with 90% ethanol,

resulting in final concentrations from 100 to 0 mg/mL, respectively. A volume of 0.10 mL of standardized inoculum was added to LB agar medium at 60 ℃. The diluted extract solution was added into Oxford cups. The experimental methods and culture conditions were identical to those described in the "Inhibition zone diameters" section. The MIC was determined as the concentration at which the extract completely inhibited the growth of the selected bacteria.

Statistics: Data were analyzed using the IBM SPSS® software (v 19.0). Duncan's multiple range test was used to perform multiple comparisons. P<0.05 was considered as statistically significant.

RESULTS

Total phenols: Results of total phenols of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts are presented in Table 1. Results represents that *S. cuneata* extract exhibited the highest total phenolic contents with a value of 226.89 mg/mL, and phenolic contents in *S. cuneata* extract was higher as compared to that of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, and *S. flavescens* (P<0.05). The *C. herba* extract also demonstrated a notable total phenolic content with a value of 98.42 mg/mL, which was significantly higher than the extracts of walnut green husk, *C. spinosa, E. prostrata, S. flavescens* and *S. barbata* (P<0.05). The lowest total phenolic contents among examined extracts was observed in the *C. spinosa* extract with a value of only 5.45 mg/mL, which was significantly lower than all other extracts (P<0.05).

Flavonoids: Results of flavonoids of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts are presented in Table 1. Results of flavonoid contents explored that the *S. cuneata* extract had highest flavonoid content (428.04 mg/mL) as compared to that of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, and *S. flavescens*. There was a significant difference between the *S. cuneata* extract and walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, and *S. flavescens* extracts (P<0.05). Results also showed that *C. herba* extract exhibited the second highest flavonoid content (201.86 mg/mL), while the extracts of walnut green husk, *S. flavescens*, and *S. barbata* showed similar flavonoid contents (P>0.05). Similar to the total phenolic content results, *C. spinosa* extract showed the lowest flavonoid content, which was significantly lower than all the other extracts (P<0.05) (Table 1).

DPPH free radical scavenging capacity: Results of DPPH free radical scavenging capacity of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts are presented in Table 1. Results of current study showed that *S. cuneata* and *E. prostrata* extracts had significantly higher DPPH free radical scavenging rate than the other extracts (P<0.05). The order of DPPH free radical scavenging capacity for the other extracts was *C. herba* > *S. barbata* > walnut green husk > *S. flavescens* > *C. spinosa*. Results of DPPH free radical scavenging capacity of the seven extracts show that *C. spinosa* extract exhibited the lowest DPPH free radical scavenging rate.

Table 1. Total phenols, flavonoids and DPPH free radical scavenging rate of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts

Extracts	Total phenols (mg GAE/mL)	Flavonoids (mg RE/mL)	DPPH free radical clearance rate (%)
Walnut green husk	21.40±1.33 ^{e}	40.96±2.53 ^{d}	50.75±0.67 ^{d}
C. herba	98.42±5.84 ^{b}	201.86±13.97 ^{b}	78.25±0.51 ^{b}
C. spinosa	5.45±0.16 ^{f}	7.34±0.51 ^{e}	28.25±2.64 ^{f}
E. prostrata	39.15±1.05 ^{cd}	137.99±10.03 ^{c}	85.33±1.51 ^{a}
S. flavescens	36.33±2.89 ^{d}	32.93±5.76 ^{d}	34.06±0.93 ^{e}
S. barbata	46.06±1.30 ^{c}	50.50±2.81 ^{d}	74.38±2.18 ^{c}
S. cuneata	226.89±9.53 ^{a}	428.04±33.34 ^{a}	83.16±1.48 ^{a}
P value	<0.001	<0.001	<0.001

Note: Results are presented as mean ± standard deviation. In the same column, values without or with the same letter superscripts indicate no significant difference ($P>0.05$), while values with different letter superscripts indicate significant difference ($P<0.05$). GAE, gallic acid equivalent; RE, rutin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

IZDs: Results of inhibition zone diameters (mm) of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts against *S. aureus*, *E. coli*, and *S. enteritidis* are presented in Table 2. Results showed that flavomycin exhibited significant antibacterial activity against *S. aureus*, *E. coli*, and *S. enteritidis*. Results of current study explored that walnut green husk extract showed the strongest inhibitory effect,

with a significantly higher IZD against *S. aureus* compared to the other extracts (P<0.05). The extracts of *E. prostrata* and *S. flavescens* also showed favorable antibacterial capabilities against *S. aureus*, with significantly larger IZDs than the extracts of *C. herba*, *C. spinosa*, and *S. cuneata* (P<0.05). However, *S. barbata* extract failed to exhibit any discernible antibacterial activity against *S. aureus* (Table 2, and Figure 1a, 1d, 1g, 1j, 1p, 1s).

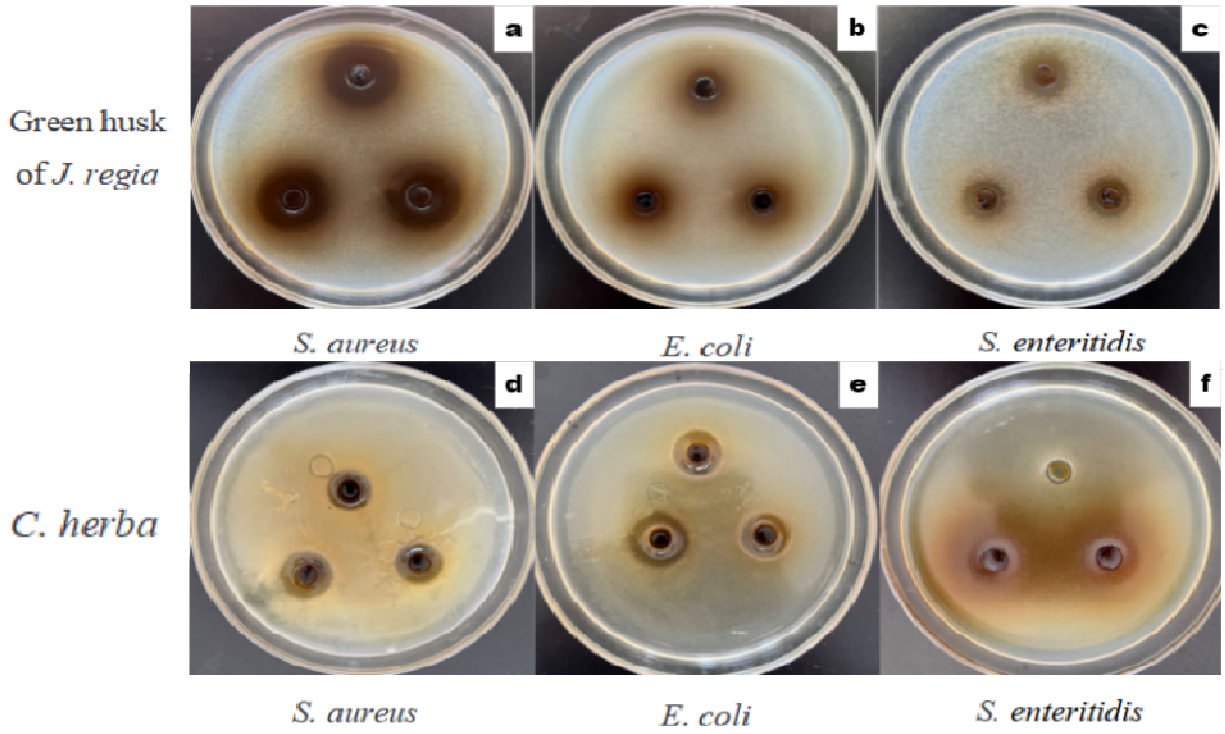
Results also showed that against *E. coli*, *S. barbata* extract was the most effective among the other six herbal extracts, and significantly surpassing the other six extracts in terms of IZD (P<0.05). Against *E. coli*, extracts of *C. herba*, *C. spinosa*, and *E. prostrata* were close, while had larger IZDs as compared to that of walnut green husk and *S. flavescens* (P<0.05). The extract of *S. cuneata* showed the weakest inhibitory effect against *E. coli*, with an IZD of only 4.40 mm (Table 2, and Figure 1b, 1e, 1h, 1k, 1q, 1t).

It was also observed that walnut green husk extract exhibited the strongest inhibitory effect against *S. enteritidis*, with a significantly larger IZD even compared to flavomycin (P<0.05). The extracts of *C. spinosa*, *E. prostrata*, and *S. barbata* showed similar antibacterial activities against *S. enteritidis*, with significantly larger IZDs compared to the extracts of *C. herba*, *S. flavescens*, and *S. cuneata* (P<0.05). However, no inhibitory effect was observed for the extracts of *C. herba* and *S. flavescens* against *S. enteritidis* (Table 2, and Figure 1c, 1f, 1i, 1l, 1r, 1u).

Table 2. Results of inhibition zone diameters (mm) of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts against *S. aureus*, *E. coli*, and *S. enteritidis*

Extracts	*S. aureus*	*E. coli*	*S. enteritidis*
Walnut green husk	13.05±0.42 ^{b}	5.84±0.34 ^{d}	8.59±0.67 ^{a}
C. herba	5.93±0.51 ^{d}	7.20±0.82 ^{c}	0.00±0.00 ^{e}
C. spinosa	5.67±0.29 ^{d}	7.17±0.76 ^{c}	5.00±1.00 ^{c}
E. prostrata	8.17±0.29 ^{c}	6.03±0.25 ^{cd}	4.67±0.29 ^{c}
S. flavescens	8.50±0.50 ^{c}	5.67±0.29 ^{d}	0.00±0.00 ^{e}
S. barbata	0.00±0.00 ^{e}	9.83±0.29 ^{b}	4.27±0.25 ^{c}
S. cuneata	5.17±0.76 ^{d}	4.40±0.17 ^{e}	1.77±0.25 ^{d}
Flavomycin	17.33±0.58 ^{a}	11.33±1.53 ^{a}	6.67±0.58 ^{b}
P value	<0.001	<0.001	<0.001

Note: Results are presented as mean ± standard deviation. In the same column, values without or with the same letter superscripts indicate no significant difference ($P>0.05$), while values with different letter superscripts indicate significant difference ($P<0.05$).



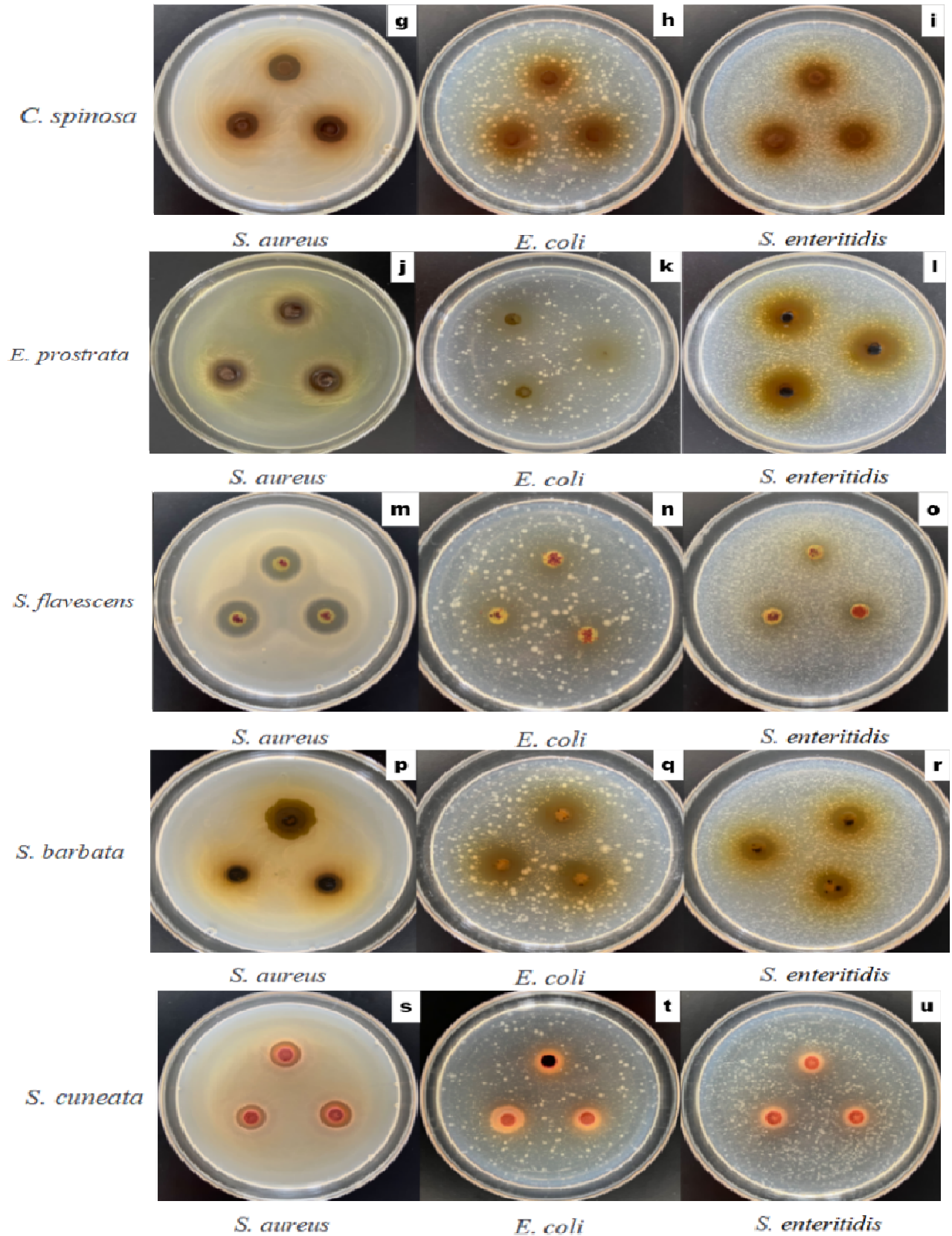


Figure 1. Antibacterial effects of walnut green husk, *C. spinosa, C. herba, E. prostrata, S. barbata, S. cuneata,* and *S. flavescens* extracts against *S. aureus, E. coli,* and *S. enteritidis*

MIC: Minimum inhibitory concentration of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts on *S. aureus* is presented in Table 3. Results indicates that the MIC of walnut green husk extract against *S. aureus* was 25 mg/mL. The extracts of *E. prostrata* and *S. flavescens* demonstrated a MIC of 50 mg/mL against *S. aureus*. The extracts of C. *herba*, *C. spinosa*, and *S. cuneata* exhibited a MIC of 100 mg/mL. However, no discernible antibacterial activity was observed for the *S. cuneata* extract at or below a concentration of 100 mg/mL.

Table 3. Minimum inhibitory concentration of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts on *S. aureus*.

Extract	Extract concentration (mg/mL)								
	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39
Walnut green husk	+	+	+	-	-	-	-	-	-
C. herba	+	-	-	-	-	-	-	-	-
C. spinosa	+	-	-	-	-	-	-	-	-
E. prostrata	+	+	-	-	-	-	-	-	-
S. flavescens	+	+	-	-	-	-	-	-	-
S. barbata	-	-	-	-	-	-	-	-	-
S. cuneata	+	-	-	-	-	-	-	-	-

Note: "+" indicates that at this concentration, it has inhibitory effect on the tested bacteria, "-" means no inhibitory effect.

Results also showed that extracts of walnut green husk, *S. flavescens*, and *S. cuneata* exhibited a MIC of 100 mg/mL against *E. coli* (Table 4). Additionally, the extracts of *C. herba*, *C. spinosa*, and *E. prostrata* displayed a MIC of 50 mg/mL, indicating a more potent antibacterial effect at this concentration level.

Table 5 results also showed that the extract from the walnut green husk demonstrated a MIC of 50 mg/mL against *S. enteritidis*. Furthermore, the extracts of *C. spinosa*, *E. prostrata*, and *S. cuneata* exhibited a MIC of 100 mg/mL. In addition, the extracts of C. *herba*, *S. flavescens*, and *S. cuneata* showed no antibacterial activity at or below a concentration of 100 mg/mL.

Table 4. Minimum inhibitory concentration of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts on *E. coli.*

Extract	Extract concentration (mg/mL)								
	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39
Walnut green husk	+	-	-	-	-	-	-	-	-
C. herba	+	+	-	-	-	-	-	-	-
C. spinosa	+	+	-	-	-	-	-	-	-
E. prostrata	+	+	-	-	-	-	-	-	-
S. flavescens	+	-	-	-	-	-	-	-	-
S. barbata	+	+	-	-	-	-	-	-	-
S. cuneata	+	-	-	-	-	-	-	-	-

Note: "+" indicates that at this concentration, it has inhibitory effect on the tested bacteria, "-" means no inhibitory effect.

Table 5. Minimum inhibitory concentration of walnut green husk, *C. spinosa*, *C. herba*, *E. prostrata*, *S. barbata*, *S. cuneata*, and *S. flavescens* extracts on ***S. enteritidis.***

Extract	Extract concentration (mg/mL)								
	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39
Walnut green husk	+	+	-	-	-	-	-	-	-
C. herba	-	-	-	-	-	-	-	-	-
C. spinosa	+	-	-	-	-	-	-	-	-
E. prostrata	+	-	-	-	-	-	-	-	-
S. flavescens	-	-	-	-	-	-	-	-	-
S. barbata	+	-	-	-	-	-	-	-	-
S. cuneata	-	-	-	-	-	-	-	-	-

Note: "+" indicates that at this concentration, it has inhibitory effect on the tested bacteria, "-" means no inhibitory effect.

DISCUSSION

Results of the present study showed that *S. enteritidis*, *S. aureus,* and *E. Coli* were inhibited by walnut green husk extract and *S. enteritidis* and *S. aureus* were being the most sensitive. It has been reported in the previous study that walnut green husk extracts abundant quantity flavonoids including juglone are known to have antibacterial properties (Nour *et al*., 2013; Wang *et al*., 2016a; Wang *et al*., 2023). Previous study of Oliveira *et al*. (2008) reported that aqueous extract of walnut green husk exhibited antibacterial property against several Gram-positive bacteria. A recent study, has demonstrated that skin wound infection caused by *S. aureus* in mouse model is cured by walnut husk extract and juglone compound in the walnut husk inhibit bacterial growth by disrupting cell membrane integrity, reducing membrane permeability, and causing protein leakage (Wan *et al*. 2023). It has also been reported that flavonoids can interfere with the bacterial DNA and RNA synthesis by inhibiting the replication process, or bind to enzymes involved in nucleic acid synthesis, and prevents bacterial replication and transcription, thereby stopping bacterial growth and proliferation (Ahmad *et al*., 2015). For example, flavonoid epigallocatechin gallate (catechin), found in green tea, inhibits DNA gyrase in *S. aureus* (Gradišar *et al*., 2007). Another study has reported that quercetin, a common flavonoid, has been known to disrupt the cell membranes of *E. coli*, leading to increased membrane permeability (Wang *et al*., 2018). Similarly, polyphenol in walnut husk may also exert antibacterial activities (Coppo and Marchese, 2014). In short, flavonoids and polyphenols are the most abundant polyphenols in plants, and they exert antibacterial activity by causing structural and organizational disruptions in bacterial cell membranes (Veiko *et al.*, 2023). Therefore, in the current study, the higher antibacterial activity due to walnut green husk could be attributed to higher flavonoid and phenolic compound present in its extract. Furthermore, it could also be assumed that some flavonoid like juglone and quercetin in walnut husk may have potential to overcome the issues of antibiotic resistance.

In previous study, over 100 compounds from *C. herba*, including iridoids, phenylethanoid glycosides (PhGs), polysaccharides, and lignans were identified (Fu *et al*., 2018). Most of the previous studies on *C. herba* examined its characteristic as antioxidant, immunity booster, against diabetes and various issues. For example, PhGs, as one of the major active compounds, have been shown to effectively inhibit the activity of aldose reductase, which may contribute to the potential for preventing or treating diabetes complications (Morikawa *et al*., 2019). Similarly, polysaccharides in *C. herba* can regulate immune activity and exert antioxidant effects (Cheng *et al*., 2023). Another study of Li *et al*. (2016) found that the acteoside-rich fraction of *C. salsa* (one of the *Cistanche* species) extract had inhibitory effects on reactive oxygen species, protected against DNA damage, increased superoxide dismutase (SOD) activity, prevented lipid peroxidation, and showed favorable antioxidant properties. Echinacoside (ECH), a phenylethanolic compound isolated from *C. herba*, has been confirmed to inhibit the proliferation of pancreatic cancer cells by inducing reactive oxygen species production, disrupting mitochondrial membranes, and finally leading to cell apoptosis (Wang *et al*., 2016b). To date, there are no reports on the antimicrobial effects of *C. herba* extract. The present study is the first to discover that *C. herba* extract possesses certain antibacterial capabilities against two bacteria of *S. aureus* and *E. coli*.

The major compounds in *C. spinosa* involve flavonoids, thioglucosides, phenolic acids, and alkaloids and they are well known for their health boosting, disease treatment and antibacterial activities. A recent study has demonstrated that thioglucosides have been shown to have excellent prevention and treatment properties against several tumors (Sun *et al*., 2023). Previous study of Boga *et al*. (2011) reported that aqueous extracts of *C. spinosa* root did not have inhibitory effect on *E. coli*, however, another study showed that the aqueous extracts of *C. spinosa* root showed certain inhibitory effects on bacteria including *S. aureus*, *E. coli*, *S. Typhimurium*, and *B. subtilis*, as well as some fungi (Zhang *et al*., 2018). Plant polysaccharides have biological activities such as antimicrobial, antioxidant, and free radical scavenging and study of Mazarei *et al*. (2017) found that the polysaccharides in *C. spinosa* leaves exhibit excellent antibacterial effects against *E. coli*, *S. Typhi*, and *S. dysenteriae*. The findings of the current study also confirmed that *C. spinosa* extract exhibited a broad-spectrum antibacterial activity, not only against *S. aureus* and *E. coli*, but also against *S. enteritidis*. This broad-spectrum antibacterial activity could be attributed to flavonoids, phenolic compounds in *C. spinosa* extract due to antioxidant capacity of the extract.

The results of this experiment also explored that *E. prostrata* extract contains abundant total phenolics and flavonoids, with a higher DPPH radical scavenging activity. These findings are similar with the findings of previous research who reported that *E. prostrata* contains coumarin derivatives, triterpenoid saponins, steroids, steroidal alkaloids, flavonoids, thiophene derivatives, and numerous other compounds (Chung *et al.*, 2017). Yadav *et al.* (2017) found that ethanol extract of *E. prostrata* exhibited potent *in vitro* antioxidant activity with high levels of total phenolics and flavonoids. In another experiment it was reported that TiO2 nanoparticles modified by *E. prostrata* extract showed favorable antibacterial activities against bacteria such as *E. coli*, as well as *S. aureus* and *Streptococcus mutans* (Maheswari *et al.*, 2020). Furthermore, previous study has also

reported that thiophene derivatives in *E. prostrata* showed moderate antibacterial activity against *S. aureus* (Yu *et al.*, 2020). Moreover, Timalsina *et al.*, (2021) reported that wedelolactone (one of the coumarin derivatives) and alkaloids of *E. prostrata* showed promising antibacterial properties. In the current study, the results suggest that *E. prostrata* extract exhibits a higher DPPH radical scavenging activity could be attributed to higher phenolic compounds (Shi *et al.*, 2022).

S. flavescens was another herb that was used in the current study. It has been reported that the active components of *S. flavescens* primarily consist of flavonoids, oxymatrine, kurarinone, and sophoridine and contents and type of these compounds varies in different parts of the plant (Sun *et al.*, 2022b). Similar with the previous herbs extract, *S. flavescens* contains antibacterial properties. A recent study by Zhang *et al*. (2023) reported that *S. flavescens* extract exhibited the strongest antibacterial activity in tilapia infected with *S. agalactiae* due to presence of bioactive compounds oxymatrine and xanthohumol. Another recent study about *S. flavescens* extract has reported that its extract contains kurarinone and sophoraflavanone G present that have potential to disruption of cell membranes, inhibition of cell wall synthesis, interference with energy metabolism, and hence inhibit methicillin-resistant *S. aureus* (Weng *et al*., 2023). In this study, *S. flavescens* extract exhibited significant antibacterial effect against *S. aureus*, while no antibacterial activity was observed against *S. enteritidis*. This discrepancy in results may be attributed to the different cellular structures of these two bacteria.

S. barbata is widely used in traditional Chinese medicine for its heat- and toxin-removing properties. Previous study has reported that 84 compounds in *S. barbata*, with flavonoids and terpenoids being the predominant components, followed by polysaccharides, volatile oils, and steroids (Chen *et al*., 2020). *S. barbata* have strong antibacterial characteristic as demonstrated by study of Tsai *et al.* (2018) who investigated 30 Chinese herbs for antibacterial activity and reported that only *S. barbata* exhibited 100% *in vitro* antibacterial activity against drug-resistant *Acinetobacter baumannii*. Similarly, another study reported that *S. barbata* oil (hexahydrofarnesylacetone, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, and menthol) have varying degrees of inhibitory effects on 17 examined microorganisms (Yu *et al*., 2004). Additionally, Sato *et al*. (2000) reported that *S. barbata* contained flavonoids have significant antibacterial activity against methicillin-resistant *S. aureus*. Nonetheless, in the current experiment, the extract of *S. barbata* did not display antibacterial activity against *S. aureus*, which may be due to variations in extraction procedures that resulted in the presence of different active compounds or level of active compound in extract, ultimately affected the experimental results.

The major components of *S. cuneata* include phenolics, phenolic glycosides, flavonoids, triterpenoids, and lignans (Zhang *et al*., 2021). In the current study, *S. cuneata* extract exhibited higher levels of total phenolics, flavonoids, and DPPH free radical scavenging rate and higher antioxidant capacity of *S. cuneata* is likely due to its high concentration of total phenolics (Li *et al*., 2008) and DPPH free radical scavenging rate. However, another study also reported that higher antioxidant status in *S. cuneata* extract could be due to its unique content of tyrosols and caffeoylquinic acids (Yang *et al.*, 2019). Zeng *et al*. (2015) isolated five novel phenolic glycosides and two dihydronaphthalene lignans from *S. cuneata* extract, along with 32 known phenolic compounds and reported that 15 compounds exhibited antibacterial activity against *S. aureus*, while two compounds demonstrated antibacterial effects against *A. baumannii in-vitro*. In the current study, *S. cuneata* extract showed some antibacterial activity against the three selected bacteria, which could be attributed to the presence of these compounds. The lower antibacterial activity observed in the current study may be due to variations in the extraction method, the solvents used, or the origin of the *S. cuneate*.

Conclusion: Based on the findings of the current study, it is concluded that walnut green husk and *S. barbata* extracts exhibit significant antimicrobial properties against *S. aureus, E. coli*, and *S. enteritidis*. Furthermore, *S. cuneata* and *E. prostrata* demonstrate strong antioxidant capacity. Given the antibiotic characteristics of walnut green husk and *S. barbata* ectracts, it is suggested that both walnut green husk and *S. barbata* have potential as natural alternatives to conventional antibiotics and open promising avenues for addressing antibiotic resistance with effective and low-toxicity treatments. However, tthe transition from in vitro studies to clinical applications involves numerous challenges, including ensuring bioavailability, safety, and efficacy in humans. Rigorous clinical trials are necessary to validate these findings and to establish these herbal extracts as viable alternatives to conventional antibiotics. Therefore, Further studies are recommended to explore their mechanisms of action and clinical applications.

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Authors' contribution: Yong Chen and Lei Wang designed the experiments; Bing-Long Chen and Ming-Xin Qiu performed the experiment; Bing-Long Chen and Lei Wang analyzed the data; Bing-Long Chen and Yong

Chen wrote the manuscript; Yong Chen and Meng-Jian Liu supervised the project; Yong Chen applied for funding. All authors approved the order of authorship and the contents of the manuscript.

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