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# Analysis and phytochemical profile of *Amaranthus tricolor* L. extract with antioxidative and antimicrobial properties

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**Abstract**

*Amaranthus tricolor* L. is a cultivated green vegetable, commonly known as amaranth, which is similar to spinach, broccoli, and cabbage. In the present study, we investigated the antioxidant and antimicrobial activities of the methanol extract and various fractions from *A. tricolor*. Results show that *A. tricolor* ethyl acetate (EtOAc) extract displayed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity ($IC_{50}$ = 16.43 ± 1.53 μg/mL), which was higher than that of the positive control, butyl hydroxy anisd ($IC_{50}$ = 19.42 ± 0.91 μg/mL). This extract had a reducing power of 2.743 at 0.5 mg/mL and significantly attenuated production of reactive oxygen species in a dose-dependent manner. Further bioassay-monitored fractionation of the EtOAc extract yielded two flavonoids, kaempferol (1) and quercetin (2), and one phenolic acid, gallic acid (3). We found that the antimicrobial activity of compound 3 (at a dose of 63 μg/mL) was superior to that of the tetracycline control (at a dose of 250 μg/mL) against *Escherichia coli*. Additionally, compounds 1 and 2 (at a dose of 63 μg/mL each) displayed higher activities against *Penicillium oxalicum* and *Staphylococcus aureus* than the control. These results suggest that *A. tricolor* extract may represent a promising nutraceutical source due to the antioxidant and antimicrobial properties of its phenolic compounds.

**Keywords:** *Amaranthus tricolor* L.; antioxidative activity; antimicrobial activity; phenolic compounds.

**Practical Application:** This article summarizes the extraction methods, biological activities and potential applications which may provide references for the follow-up research

## 1 Introduction

Free radicals, such as reactive oxygen species (ROS), are atoms or molecules that contain one or more unpaired valence electrons and are generally understood to play important roles in the development of inflammation, tissue damage, and pathological events. Conversely, stable molecules known as antioxidants can donate an electron to a chemically reactive free radical, thereby reducing its damage-inducing capacity (Wang et al., 2017). A number of antioxidants have been identified from dietary sources, particularly in plant-based foods. These natural products with antioxidant activity have numerous reported health benefits and could potentially be leveraged to prevent oxidative damage in tissue by scavenging free radicals and increasing host defenses. Plants can also produce a wide variety of secondary metabolites, including alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones, and coumarins. These biomolecules represent a rich source of plant-derived antimicrobial substances, some of which have been shown to be highly efficacious for the treatment of bacterial infections (Elisha et al., 2017).

*Amaranthus tricolor* L. is a green vegetable that is similar to spinach, broccoli, and cabbage and is widely cultivated in warm and tropical regions. Commonly known as amaranth, it is mainly consumed as a leafy vegetable (Zhang et al., 2021). Edible stems and leaves of *A. tricolor* are an inexpensive and abundant source of digestive fiber, protein containing methionine and lysine, vitamin C, carotenoids, minerals. It has also abundant antioxidant pigments, such as betacyanin, anthocyanin, betaxanthin, betalain, carotenoids, and chlorophylls (Sarker & Oba, 2020). Thus, this plant is an economically important source of vital dietary nutrients. *A. tricolor* exhibits potent biological activity *in vivo*, including reported anticancer activity and the ability to decrease glucose and labor pain, and is also a rich source of betalain pigments (Liu et al., 2019). However, few studies have investigated the antibacterial and free-radical scavenging activities of *A. tricolor*. Here, we generated *A. tricolor* extracts and measured free-radical scavenging ability against 2,2-diphenyl-1-picrylhydrazyl (DPPH), inhibition of ROS formation, and antibacterial activity. In addition, we identified putative active substances within the most potent extract, which may hold potential for use as antioxidant and antimicrobial agents.

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## 2 Materials and methods

### *2.1 Plant material*

The *A. tricolor* plants were cultivated at the botanical garden of Hezhou University for 2 months. Mature A. tricolor plants (16-24 cm in height) were harvested.

### *2.2 Extraction of samples for analysis*

The whole plant of *A. tricolor* were dried at room temperature and powdered, using a blender. The air-dried powdered was extracted three times with 100% methanol. Then, the methanol extract was filtered and then evaporated under reduced pressure using a vacuum rotary evaporator (R220. ENCO, Shanghai, China) to produce a crude extract. After that, the crude extract was suspended in distilled water and then partitioned with *n*-hexane, ethyl-acetate (EtOAc), butanol (water saturated BuOH). The extract and fractions were evaporated using a vacuum rotary evaporator. The dried samples were weighed and kept in a refrigerator at -20 °C for further analysis.

### *2.3 Determination of total phenolic and flavonoid contents*

The total phenolic content was determined using Folin-Ciocalteu method (Singleton & Rossi, 1964). Briefly, 0.1 mL of the diluted sample was reacted with 0.05 mL of Folin-Ciocalteu reagent about 4 min, and then a 0.3 mL of 20% sodium carbonate was added into the reaction mixture, the mixture was shaken immediately. Finally, 1 mL of distilled water was added after incubating at room temperature for 15 min. The absorbance reading was taken at 725 nm in triplicates, and the results were expressed as gallic acid equivalent per gram of extract by referencing the gallic acid standard calibration curve (mg GAE/g).

The total flavonoid content was determined by Park et al. (1997). An aliquot of 100 µL was added to 100 µL of 10% $Al(NO_3)_3$, 100 µL of 1 M potassium acetate, and 4.3 mL of 80% ethanol. After 40 min standing the absorbance of the reaction mixture at 415 nm. The data were expressed as mg/g quercetin.

### *2.4 DPPH free radical scavenging activity*

The free radical scavenging capacity of the extract and fractions was determined by methods of Xiong et al. (1996). In this assay, 0.1 mL of diluted sample at different concentrations was mixed with 0.1 mL of DPPH (1.1-diphenly-2-picrylhydrazol) 0.15 mM dissolve in methanol. The reaction mixture was incubated for 30 min in the dark at room temperature. The control contained all reagents without the sample, and methanol was used as the blank. Measurements were performed in three replications and the absorbance was measured at 517 nm and expressed as the inhibition percentage of free radicals was calculated as: (%) inhibition = (1 - absorbance of sample / absorbance of control) × 100. The $IC_{50}$ value (µg/mL) represents the concentration at which the scavenging activity is 50%.

### *2.5 Reducing power assay*

The reducing power of *A. tricolor* was measured according to a method of Oyaizu (1986) with some modifications. Predetermined concentrations of samples in 0.1 mL were mixed with 0.1 mL of sodium phosphate buffer (pH 6.6, 0.2 M) and 0.1 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 0.1 mL of 10% trichloroacetic acid (w/w) were added, 0.4 mL of distill water was then added and 0.05 mL of 0.1% of ferric chloride, and the absorbance was measured at 700 nm. Ascorbic acid was used for comparison.

### *2.6 Assay of ROS production*

The evolution of reactive oxygen species (ROS) using dichlorofluorescin diacetate (DCFH-DA) assay (Wang & Joseph, 1999). Briefly, $1 \times 10^4$ cells/well cells were plated into 6-well plates in PBS buffer for 30 min at 37 °C. The cells in the plates were washed twice with PBS and treated with 20 mM DCFH-DA and lipopolysaccharide (100 ng/mL). ROS levels were measured immediately by flow cytometry.

### *2.7 Antimicrobial activity assay*

Antibacterial activities of crude extract and its fractions were tested against *Asperigillus awamori* (ATCC11360), *Asperigillus niger* (ATCC36782), *Cladosporium herbarum* (ATCC26362), *Penicillium oxalicum* (ATCC1126), *Klebsiella pneumoniae* (ATCC55234), *Staphylococcus aureus* (ATCC90179) *Bacillus subtilis* (ATCC 23059-B1), and *Escherichia coli* (ATCC11303) using the two-fold dilution method (Swenson et al., 2007) with some modifications. Each strain was incubated in micrococcus, nutrient medium cultured at 30 and 37 °C, respectively. The strains were placed into medium and incubated at each temperature for 24 h. The optical densities were determined at 600 nm using a spectrophotometer. The culturing was stopped when an absorbance of 0.4 was reached. Samples were then dissolved in DMSO to produce 10 mg/mL stock solutions. In a 96-well microassay plate, 20 µL of each stock solution was incorporated into 180 µL of medium containing *A. awamori*, *A. niger*, *C. herbarum*, *K. pneumonia*, *S. aureus, P. oxalicum, B. subtilis,* or *E. coli*. Micrococcus medium at a concentration of 1 mg/mL was serially diluted by double technique to achieve a concentration ranging from 1,000 to 8 µg/mL. The culture plates were then incubated at each temperature for 24 h. Three replicates were performed for each treatment. The MIC of preparation was the lowest concentration that completely inhibited the visible bacterial growth.

### *2.8 Isolation of biological compounds*

The ethyl acetate fractions showed interesting biological activity and was therefore selected for the isolation and characterization of the main activity compound from *A. tricolor*. The EtOAc fraction (52 g) was separated on silica gel (100-200 mesh) using stepwise gradient elution with petroleum ether: ethyl acetate (80:20-50:50) to yield 5 fractions (Fr.1-Fr.5). Fr. 2 (15.5 g) was chromatographed on silica gel (300-400 mesh), and eluted with trichloromethane: methanol: acetic acid (75:15:1) to obtain 3 fractions (Fr.2-1-Fr.2-3). Recrystallization for the subfraction Fr. 2-2 yielded compound 1. Fr. 3 (20.8 g) was further purified on silica gel (300-400 mesh) using petroleum ether: ethyl acetate (75:25) to give compound 2 and compound 3.

### *2.9 Statistical analysis*

Data were statistically evaluated via Duncan's one-way analysis of variance (ANOVA). The level considered significant when $P < 0.05$. Data were presented as means ± standard errors.

## 3 Results and discussion

### *3.1 Antioxidant activity*

We investigated the antioxidant activity of *A. tricolor* extracts by measuring DPPH free radical-scavenging activity, reducing power, total phenolic and flavonoid content, and attenuation of ROS generation. Our results show that the ethyl acetate (EtOAc) fraction exhibits a stronger ability to scavenge DPPH radicals ($IC_{50}$, 16.43 ± 1.53 μg/mL) than both the other fractions and the positive control, butyl hydroxy anisd (BHT; 2,6-Di-tert-Butyl-4-methylphenol; $IC_{50}$, 80.81 ± 1.12 μg/mL) (Table 1). We further measured reducing power at various extract concentrations and found that the solvent fractions and crude extract display significantly different reducing powers, which increases with increasing concentration. Maximum reducing activity was present in the EtOAc fraction, corresponding to an absorbance of 1.785 at 0.3 mg/mL, 2.743 at 0.5 mg/mL, and 3.215 at 1 mg/mL in this analysis (Figure 1).

Consensus opinion is that antioxidant activity in plant extracts is related to the total phenolic content (TPC) and total flavonoid content (TFC). This is consistent with our results showing that the EtOAc fraction contains the highest TPC and TFC (232.41 ± 6.79 mg GAE/g and 89.22 ± 3.49 mg QE/g, respectively) compared to other fractions, whereas the aqueous fraction has the lowest TPC and TFC. Overall, these findings reveal that *A. tricolor* EtOAc extract possesses significant antioxidant activity. Similarly, a study by Yang et al. (2008) reported the polyphenol-rich EtOAc fraction from *Salvia miltiorrhiza* Bunge to be most effective at DPPH free radical scavenging and inhibition of platelet function. Total phenolic and flavonoid contents in this EtOAc fraction were found to be higher than in the other fractions and are comparable to those detected in our study. This result is also consistent with a report by Sahreen et al. (2017),

**Table 1.** Total phenolic, flavonoid contents and DPPH radical scavenging activity of extract and fractions from *A. tricolor*.

| Fraction | DPPH radical scavenging activity $IC_{50}$ (μg/mL) | TPC[1] (mg GAE/g) | TFC[2] (mg QE/g) |
|---|---|---|---|
| MeOH | 61.38 ± 3.21[c] | 87.55 ± 1.46[c] | 4.11 ± 0.87[c] |
| *n*-Hexane | 98.24 ± 4.12[d] | 37.93 ± 4.77[d] | ND |
| EtOAc | 16.43 ± 1.53[a] | 232.41 ± 6.79[a] | 89.22 ± 3.49[a] |
| BuOH | 45.87 ± 1.69[b] | 103.31 ± 5.61[b] | 21.67 ± 2.82[b] |
| Aqueous | 131.87 ± 3.67[e] | 21.37 ± 4.23[d] | ND |
| BHA | 19.42 ± 0.91 | | |
| BHT | 80.81 ± 1.12 | | |

[1]Total phenolic contents. [2]Total flavonoid contents. Means values followed by the same letter in the same column are not significantly different at $p < 0.05$ by Duncan's multiple range test. ND: Not detected.

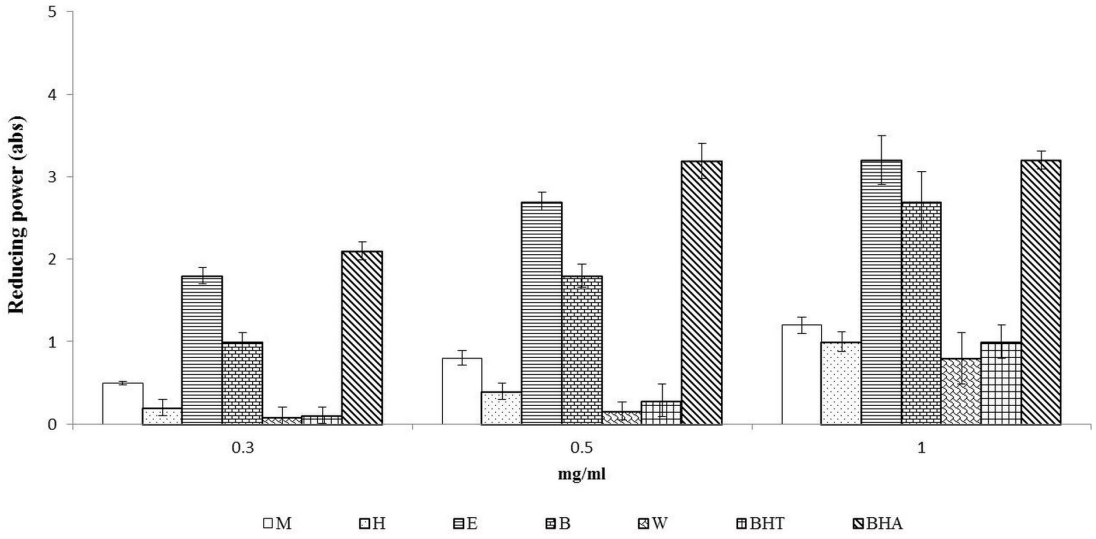


**Figure 1**. Reducing power of extract and fractions from *A. tricolor*. M: methanol extract; H: *n*-hexane fraction; E: ethyl acetate fraction; B: butanol fraction; W: water fraction; BHA: Butyl hydroxyanisole; BHT: Butylated hydroxytoluene. Data is expressed as the mean ± standard deviation of three independent experiments.

which identified a significant correlation between TPC and DPPH free radical-scavenging activity.

ROS are formed by hydrogen peroxide or superoxide anions. Critically, increased production of ROS can result in oxidative stress, leading to DNA damage and destruction of cells and tissues (Liu et al., 2014; Maran et al., 2014). Liu & Jiang (2012) found that plants possess many non-vitamin antioxidants, which are able to prevent ROS production. Therefore, we used the ROS indicator 2′,7′-dichlorofluorescin diacetate (DCF-DA) to determine whether *A. tricolor* extracts can similarly inhibit ROS generation from Raw 264.7 cells treated with lipopolysaccharide (LPS) (Figure 2). For these experiments, untreated Raw 264.7 cells (no *A. tricolor* extracts, no DCF-DA) served as the baseline control; whereas treatment with LPS alone was used to determine maximum ROS production. We found that Raw 264.7 cells treated with the EtOAc fraction produced fewer ROS than cells treated with other fractions, crude extract, or LPS in the absence of sample extract. This inhibition of ROS formation was dose-dependent, and at the highest dose of EtOAc fraction tested (500 mg/mL), ROS production reached its lowest level. These results suggest that the EtOAc fraction can inhibit ROS production by scavenging free radicals. These effects are in agreement with a report by Ghosh et al. (2018), who found that *Aronia melanocarpa* 'Viking' extracts display strong free radical-scavenging activity and may have a crucial role in inhibiting ROS generation. Oxidative stress has been proposed as a causative factor in the toxicity of numerous chemicals, and it has a significant role in the pathogenesis of several human diseases, including coronary heart disease and cancers (Tinkel et al., 2012; Glasauer & Chandel, 2014). Thus, our findings suggest the *A. tricolor* EtOAc fraction contains antioxidants that may be used to prevent the adverse effects of oxygen reactivity by capturing and neutralizing free radicals, thereby eliminating free radical damage to the human body.

### *3.2 Antimicrobial activity*

We next measured the antimicrobial efficacy of *A. tricolor* extract and fractions against various microbes. In general, we observed that extract potency is dependent on both the solvent fraction and the test organism used. Among the extract and various fractions tested, we found that the EtOAc fraction displayed remarkable antimicrobial activity against all fungi and bacterial strains that were assessed (Table 2). However,

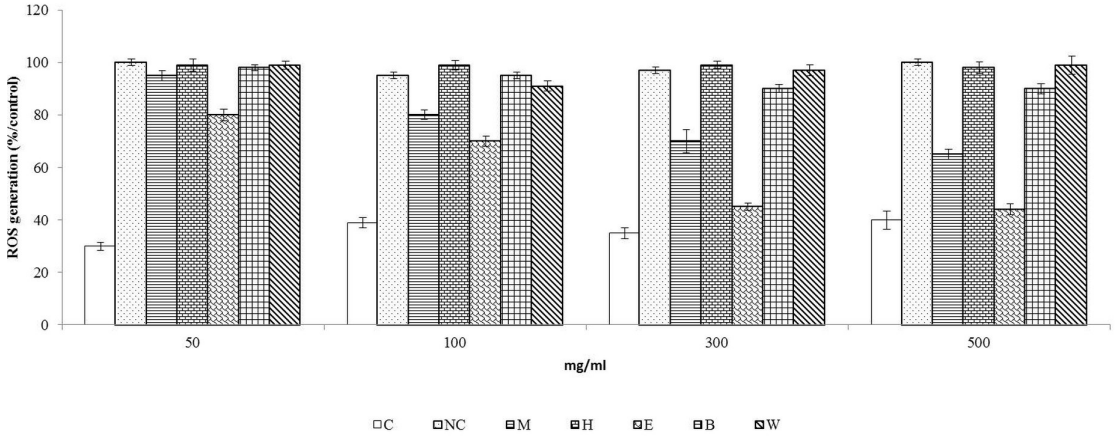


**Figure 2**. Effect of *A. tricolor* extract on ROS generation in RAW 264.7 cells. C: control; NC: negative control; M: methanol extract; H: *n*-hexane fraction; E: ethyl acetate fraction; B: butanol fraction; W: water fraction.

**Table 2.** Antimicrobial activity of *A. tricolo* extract *r*, its fractions and isolated compounds.

| Tested samples | MIC (μg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Fungi strain | | | | Bacteria strain | | | |
| | *A. awamori* | *A. niger* | *C. herbarum* | *P. oxalicum* | *K. pneumonia* | *S. aureus* | *B. subtilis* | *E. coli* |
| MeOH extract | 250 | 125 | 250 | 125 | 125 | 63 | 32 | 125 |
| Hexane fraction | 500 | 500 | 250 | 1000 | 1000 | 1000 | 1000 | 1000 |
| EtOAc fraction | 125 | 125 | 125 | 63 | 125 | 32 | 63 | 125 |
| BuOH fraction | 250 | 500 | 250 | 125 | 500 | 32 | 250 | 125 |
| Water fraction | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |
| Compound 1 | 250 | 250 | 500 | 63 | 250 | 63 | 250 | 250 |
| Compound 2 | 125 | 125 | 250 | 63 | 125 | 63 | 125 | 125 |
| Compound 3 | 125 | 63 | 125 | 8 | 63 | 16 | 8 | 63 |
| Tetracycline | | | | | 25 | 8 | 8 | 250 |
| Cyclohexamide | 4 | 4 | 25 | 25 | | | | |

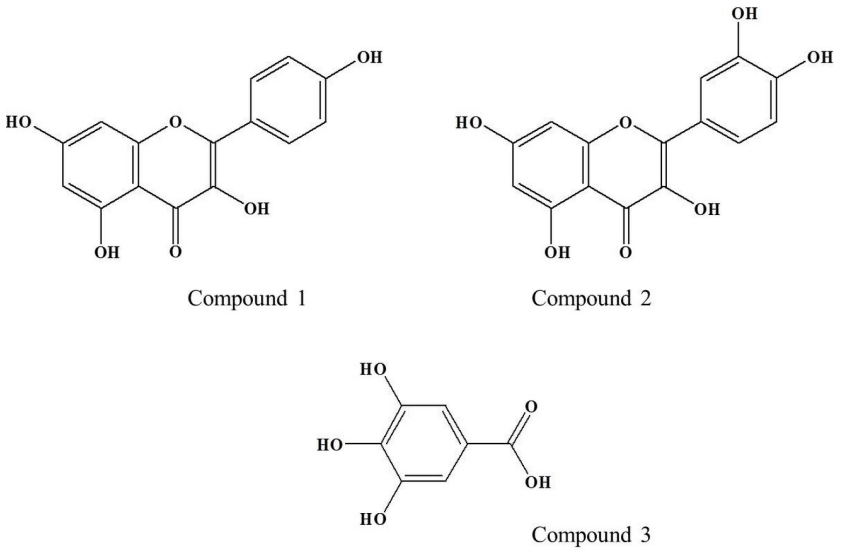


**Figure 3**. Chemical structures of the isolated compounds.

the butanol (BuOH) fraction showed the highest antibacterial activity against *Staphylococcus aureus* and *Escherichia coli,* as compared with all other fractions tested (Table 2). These data indicate that *A. tricolor* fractions contain compounds with potent antimicrobial activity.

### *3.3 Isolated compounds from the EtOAc fraction of A. tricolor*

To further determine the source of this antimicrobial activity, we performed a bioassay-guided fractionation of the most active EtOAc fraction, which led to the isolation of three compounds: kaempferol (Compound 1), quercetin (Compound 2), and gallic acid (Compound 3). Compounds 1 and 2 are flavonoids, and Compound 3 is a phenolic acid. All structures were confirmed by electrospray ionization-mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) (Figure 3). We then measured antimicrobial activities of the isolated compounds, and as shown in Table 2, all three compounds showed substantial activities against the test organisms. Among them, gallic acid showed the highest activity against *Aspergillus niger*, *Klebsiella pneumonia*, *E. coli*, *S. aureus, Penicillium oxalicum,* and *Bacillus subtilis*, with minimum inhibitory concentration (MIC) values of 63-8 μg/mL. In addition, we further found that kaempferol and quercetin exhibit fairly strong antibacterial activity toward *P. oxalicum* and *S. aureus*.

Phenols and flavonoids are known to be important secondary plant products found in foods and medicinal plants. Various biological and pharmacological activities have been attributed to these compounds, including anticancer, antispasmodic, and anti-inflammatory effects (García-Mediavilla et al., 2007). Plants also synthesize phenols as a chemical defense system against predation by herbivores, insects, and microorganisms (Feng & Xu, 2014). Accordingly, recent studies have described plant-derived compounds with antimicrobial properties that may be used to inhibit the growth of pathogenic microorganisms and act as therapeutic agents for the treatment of infectious diseases. Moreover, these compounds could kill or inhibit growth of pathogenic microorganisms via a distinct mechanism (Chew et al., 2018).

Gallic acid is a well-known antimicrobial compound, which is considered to be safe in nature; it is widely present in the human diet and is often used by the food industry as a flavoring agent. Moreover, gallic acid is found in nearly every part of the plant, including the bark, wood, leaf, fruit, root, and seed. This compound is also present in different concentrations in common foodstuffs, such as blueberries, blackberries, strawberries, plums, grapes, mangos, cashew nuts, hazelnuts, walnuts, tea, and wine (Daglia et al., 2014). Notably, given its wide availability, the findings from our study further emphasize the potential of gallic acid as a natural and sustainable source of new broad-spectrum antimicrobial products. The antimicrobial activities of quercetin and kaempferol are also intriguing, given that these flavonoid compounds, which are found in many herbal extracts and have strong antioxidative capacity, have been proposed as natural agents for cancer prevention and antimicrobial treatment (Chen & Chen, 2013). Based on these findings and results from our present study, *A. tricolor* extracts may be used as a raw material to formulate promising new products for the food industry, including effective natural antibacterial preservatives.

## 4 Conclusion

In summary, results of the present study provide evidence for antioxidative and antimicrobial activities in plant extracts from *A. tricolor* and further reveal the identities of these bioactive compounds. We show that *A. tricolor* EtOAc extract possesses potent DPPH free radical-scavenging and antimicrobial activities, as well as high phenolic and flavonoid contents and the ability to significantly reduce ROS production in a dose-dependent manner. Bioactivity-guided fractionation of the most active EtOAc

fraction led to the isolation of antimicrobial three compounds: kaempferol, quercetin, and gallic acid, although further work will be required to determine their precise mode of action. From these findings, we conclude that extracts of *A. tricolor* have a significant potential for use as natural antibacterial preservatives.

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