Kragujevac J. Sci. 41 (2019) 107-116. UDC 579.26:581.6:582.548.27:582.711:615.322
Original scientific paper

SYNERGISTIC ANTIBACTERIAL ACTIVITY OF *Curcuma longa* L. AND *Urtica dioica* L. EXTRACTS AND PRESERVATIVES

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(Received March 29th, 2019; Accepted May 10th, 2019)

ABSTRACT. *Curcuma longa* L. (turmeric) and *Urtica dioica* L. (common nettle) are well-known medicinal plants. In recent decades, due to its excellent biological activity and low toxicity, significant efforts have been made to research the potential application of these plants. Accordingly, the aim of this study was to investigate the combined effects of preservatives (potassium nitrite and sodium nitrite) and ethanol, acetone, and ethyl acetate extracts of *C. longa* and *U. dioica* emphasizing their join, synergistic effects. The eight isolates of foodborne pathogenic bacteria and two reference strains were used. The interactions were tested by the checkerboard method and expressed as fractional inhibitory concentration (FIC) index. FIC index was ranged in intervals from 0.25 to 2.0. The extracts of *C. longa* showed better synergistic effect with preservatives than *U. dioica* extracts. Synergism was observed in relation to *Staphylococcus aureus* ATCC 25923, *S. aureus*, *Bacillus subtilis*, and *Salmonella typhimurium*. In the presence of sub-inhibitory concentrations (1/4 MIC, 1/8 MIC) of the extracts, the MICs of preservatives have decreased up to 8-fold. In addition, the contents of phenolic compounds (total phenols, flavonoids, tannins, and proanthocyanidins) were determined spectrophotometrically. The higher concentration of phenolic compounds was detected in *C. longa* extracts than in *U. dioica* extracts.

Keywords: *Curcuma longa*, *Urtica dioica*, preservatives, antibacterial activity, synergistic effect.

INTRODUCTION

The great need for high-quality and safe food requires new, natural methods of long-term storage of food. This presents significant challenges, especially because there is growing disagreement regarding the use of chemical preservatives and artificial antimicrobial agents. There are many studies that have shown that preservatives can cause allergies, increase the risk of cancer, affect negatively on children's health and can cause hyperactivity in children (SINHA *et al*., 2005; JAKSZYN *et al*., 2006; MCCANN *et al*., 2007). Therefore, in recent years, the natural antimicrobial compounds isolated from plants have been gaining in importance. These compounds control the microbial contamination of food inhibiting the

growth of microorganisms, increase the duration of canned food, and they are recognized as safe for human health (TAJKARIMI *et al*., 2010).

Plants produce bioactive substances which arise as a secondary metabolism products which exhibit antimicrobial, antioxidant, antiinflammatory, anticancer and other biological properties. Thanks to their bioactive metabolites, some plants have been used for a long time in traditional medicine, the effect of which has been confirmed by scientific researches. The bioactive substances isolated from the plants are classified in the three groups: phenolic compounds (phenols, flavonoids, tannins, coumarins, proanthocyanidins), alkaloids and terpenoids (COWAN *et al*., 2010).

The species *Curcuma longa* L. (Zingiberaceae) – turmeric is a well-known spicy and medicinal plant. It is native to India, China, but it is today also cultivated in Sri Lanka, Indonesia, Bangladesh, Burma, and Pakistan (GOVINDARAJAN, 1980). It is most commonly used in cooking and food industry, traditional medicine, pharmacy, etc. Moreover, the curcuma powder is commercial available as a spice, as an ingredient in curries, mustard, as a flavoring agent (PRASAD *et al*., 2014), as well as a herbal supplement for variety of human diseases (GHOSH *et al*., 2015; SHANBHAG, 2017; LELLI *et al*., 2017; SRIVASTAV *et al*., 2017; RAUF *et al*., 2018). A large number of papers about the *C. longa* plant indicate its importance in medicine, and the most active compound isolated from the rhizomes of this plant is curcumin (HUANG *et al*., 2018). In addition, turmeric is used in some countries for the preservation of foods because of its antimicrobial activity (JAYAPRAKASHA *et al*., 2005).

The species *Urtica dioica* L. (Urticaceae) – common nettle is used in traditional medicine as a tonic, in addition, this plant also has a mild diuretic effect and helps the elimination of toxins. Fresh, young leaves contain minerals, especially selenium, calcium, magnesium, zink and iron, and vitamins and because of their nutritional value, *U. dioica* is used as food (RUTTO *et al*., 2013; RAFAJLOVSKA *et al*., 2013). Clinical studies suggest that *U. dioica* contain compounds that affect the hormones responsible for benign prostatic hyperplasia (KOCH, 2005). In addition, common nettle can help alleviate the symptoms of osteoarthritis and joint pain (RANDALL *et al*., 2000), rheumatoid arthritis (KLINGELHOEFER *et al*., 1999). Infusions of the plant can be used for nasal and menstrual hemorrhage, diabetes, anemia, asthma, hair loss and to promote lactation (KHARE *et al*., 2012). Moreover, common nettle also shows antibacterial properties (KREGIEL *et al*., 2005). The species *U. dioica* is native to Europa, North Africa, North America and in continental parts of Asia (KREGIEL *et al*., 2005).

In our earlier paper antibacterial activity of ethanol, acetone and ethyl acetate extracts of *C. longa* and *U. dioica* was reported (STEFANOVIĆ *et al*., 2019). As further steps in antibacterial testing, the aim of this study was an evaluation of the synergistic effect of preservatives and bioactive metabolites of *C. longa* and *U. dioica* with the purpose that reducing the use of synthetic preservatives.

MATERIAL AND METHODS

Preparation of samples for testing

The *Urtica dioica* dried ground leaves and *Curcuma longa* dried rhizomes powder, supplied from the commercial source, were used in extraction. The plant material was extracted by maceration with ethanol, ethyl acetate, and acetone for three days at room temperature. Every 24 h, 20 g of plant material was soaked with a new amount (150 mL) of the appropriate solvent. After filtration, the extracts were concentrated using a rotary evaporator (IKA, Germany) at 40°C to obtained dry extracts without a trace of solvent. Before

the testing, the extracts were dissolved in dimethyl sulfoxide (DMSO) and then diluted into a nutrient liquid medium to achieve a concentration of 10% DMSO.

Two preservatives, sodium nitrite (E – 250) and potassium nitrite (E – 249) (Alkaloid, Macedonia) were used. Stock solutions of preservatives were prepared in Mueller-Hinton broth (Torlak, Serbia).

Bacteria

A total of 10 strains of bacteria were used, including 8 isolates (*Staphylococcus aureus* (a dairy product), *Bacillus subtilis* (an environmental origin), *Escherichia coli* (a tea - dried plant material), *Escherichia coli* O157 (a dairy product), *Klebsiella oxytoca* (a dairy product), *Proteus mirabilis* (a fresh meat), *Salmonella enterica* (an egg), *Salmonella typhimurium* (an egg), and 2 reference strains (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923). The density of the bacterial suspension was measured by the apparatus – densitometer (BIOSAN, Latvia). For standardization of bacterial suspensions and determination of the number of bacteria, Mc Farland standard number 0.5 was used, which indicates the number of bacteria of 1.0 - 1.5 × 10^8 colony forming units per mililiter (CFU/mL). Ten-fold dilutions of initial suspension were additionally prepared into sterile 0.85% saline to achieve 1.0 × 10^6 CFU/mL. The final concentration of bacteria into the wells was 1.0 × 10^5 CFU/mL.

Phytochemical analysis of plant extracts

The contents of phenolic compounds were determined spectrophotometrically. The total phenol content was detected with Folin-Ciocalteu reagent (WOOTTON-BEARD *et al*., 2011) and total flavonoid content with 2% aluminum chloride (QUETTIER-DELEU *et al*., 2000) and results were expressed as gallic acid equivalent (mg of GA/g of extract) and rutin equivalent (mg of RU/g of extract), respectively. The total extractable tannin content was estimated indirectly by spectrophotometric measurement of the absorbance of the solution obtained after the precipitation of the tannins with polyvinylpolypyrrolidone as described by MAKKAR *et al*. (1993). The total extractable tannin content was expressed as gallic acid equivalent (mg GA/g of extract). The proanthocyanidin content was measured by the butanol-HCl method with ferric ammonium sulfate as a catalyst as described by PORTER *et al*. (1986). Cyanidin chloride was used as the standard and the proanthocyanidin content was expressed as cyanidin chloride equivalent (mg CCh/g of extract). All chemicals and reagents were purchased from Sigma-Aldrich, USA.

Combination assay

Prior to performing the synergy test, the minimum inhibitory concentrations (MICs) of plant extracts and preservatives were determined using microdilution plate method with resazurin as described in STEFANOVIĆ *et al*. (2019).

The combined activity of plant extracts and preservatives was evaluated by the checkerboard method (SATISH *et al*., 2005). The testing was performed in 96-well microtiter plates using a 4-by-4 well configuration. Two-fold dilutions of each antibacterial agents (plant extracts and preservatives) were prepared to achieve the final concentration range corresponded to 1/8 MIC – MIC. Firstly, 50 μL of each dilution of plant extract was added horizontally into four rows and, then, 50 μL of each dilution of preservative was added vertically into four columns. The final volume was 100 μL. Each well contained a unique combination of plant extract and preservative concentration. Ten microliters of each 1.0 × 10^6 CFU/mL bacterial suspension and 10 μL of resazurin solution was added. The microtiter plates were incubated for 24 h at 37°C. The combination of the compounds in which resazurin

color change did not appear (growth inhibition) is taken as effective MIC for the combination. Each test included growth control and medium-sterility control. In addition, the control of solvent (10% DMSO) was prepared. The 10% DMSO did not inhibit the growth of tested bacteria and, also, in the experiment the concentration of DMSO was serially decreased.

In vitro interactions between antimicrobial agents were determined and quantified by calculating the fractional inhibitory concentration index (FIC index) using the formula (1):

$$\text{FIC index} = \frac{\text{MICa in comb.}}{\text{MICa alone}} + \frac{\text{MICb in comb.}}{\text{MICb alone}} \quad (1)$$

Where MICa – MIC of tested extract and MICb - MIC of tested preservative.

Based on the value of FIC index, categorization of common effects can be performed as follows: FIC index ≤ 0.5 – indicate synergistic effect; FIC index > 0.5 – 1 indicate additive effect; FIC index > 1 – 4 indicate indifferent effect; FIC index > 4 indicate antagonistic effect. FIC index was calculated for each combination in a 4 × 4 configuration that inhibited bacterial growth (SATISH *et al*., 2005; MELETIADIS *et al*., 2010).

RESULTS AND DISCUSSION

Total phenols, flavonoids, tannins, and proanthocyanidins in the extracts of plant species

The measured concentrations of total phenols, total flavonoids, tannins and proanthocyanidins in ethanol, acetone and ethyl acetate extracts of *Curcuma longa* and *Urtica dioica* are shown in Table 1. It can be seen that the higher concentration of phenolic compounds was detected in *C. longa* extracts than in *U. dioica* extracts. *C. longa* ethyl acetate extract contented the highest quantity of phenolic compounds, hence, ethyl acetate could be a solvent of choice for extraction of bioactive compounds from *C. longa* plant material. The main components of *C. longa* are curcuminoids which belong to the group of phenolic substances. The curcuminoids, are a mixture of three principal compounds: curcumin (77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%). The content of curcuminoids especially curcumin is used as one of the parameters in quality control of *C. longa* and the therapeutic effects are associated with the level of curcumin. Therefore, the chemical composition of *C. longa* extracts was directed to determination and isolation of curcuminoids (JAYAPRAKASHA *et al*., 2005; HUANG *et al*., 2018).

Table 1. The concentration of total phenols, flavonoids, tannins and proanthocyanidins in extracts of *Curcuma longa* and *Urtica dioica*.

Plant species	Type of extract	Total phenols (mgGA/g extract)	Flavonoids (mgRU/g extract)	Tannins (mgGA/g extract)	Proanthocyanidins (mgCCh/g extract)
C. longa	Ethanol extract	147. 58	35. 79	118. 49	6. 28
	Acetone extract	159. 74	95. 79	120. 56	3. 99
	Ethyl acetate extract	208. 98	85. 77	172. 69	6. 21
U. dioica	Ethanol extract	17. 04	55. 71	10. 54	5. 55
	Acetone extract	19. 88	50. 23	16. 89	5. 22
	Ethyl acetate extract	15. 96	44. 51	22. 16	5. 00

In our study, *U. dioica* extracts were rich in flavonoids. On the other side, KUKRIĆ *et al*. (2012) found that the total phenolic content in 80% ethanol extract of *U. dioica* was 208.37 mg GA/gdw, while total flavonoid content was 20.29 mg Q/gdw. VAJIĆ *et al*. (2015), have reported that total phenol content in ethanol extract was 0.4 mg GA/gdw. GHAIMA *et al*. (2013) measured 48.3 mg GA/mg in ethyl acetate extract. Obviously, the content and composition of phenolic compounds depend on different factors such as the variety, genotype, climate, soil, vegetative stage, harvest time, storage condition, and extraction procedure.

Proanthocyanidins were a group of phenolic compounds detected in the lowest concentration in extracts of both plants.

Antibacterial activity of extracts of tested plant species and preservatives

The antibacterial activity of tested plant extracts was previously evaluated and the results were reported in STEFANOVIĆ *et al*. (2019). The intensity of antibacterial activity depended on the species of bacteria, plant species and the type of extract. The extracts were more active against Gram-positive bacteria than Gram-negative bacteria. Comparing the obtained results of the antibacterial activity of the tested extracts with positive control results (preservatives), it was noted that the extracts showed equal or higher activity.

Table 2. The MIC values of tested preservatives and FIC index values showing different types of interaction between *Curcuma longa* extracts and preservatives.

Bacterial strains	**MIC (mg/mL)**		**Ethanol extract**		**Acetone extract**		**Ethyl acetate extract**	
	KNO_2	**$NaNO_2$**	**KNO_2**	**$NaNO_2$**	**KNO_2**	**$NaNO_2$**	**KNO_2**	**$NaNO_2$**
S. enterica	50	12.5	2 (I)	2 (I)	2 (I)	2 (I)	2 (I)	2 (I)
S. typhimurium	50	12.5	2 (I)	1.125 (I)	2 (I)	1.125 (I)	2 (I)	1.125 (I)
***E. coli* ATCC 25922**	50	12.5	1.125 (I)	1.125 (I)	1.125 (I)	1.125 (I)	2 (I)	1.125 (I)
E. coli	50	12.5	2 (I)	1.125 (I)	2 (I)	1.125 (I)	2 (I)	1.125 (I)
***E. coli* O157**	50	12.5	2 (I)	1.125 (I)	2 (I)	1.125 (I)	2 (I)	0.625 (A)
K. oxytoca	50	12.5	2 (I)	2 (I)	2 (I)	2 (I)	2 (I)	2 (I)
P. mirabilis	100	25	1.25 (I)	0.625 (A)	2 (I)	0.625 (A)	1.125 (I)	1.125 (I)
***S. aureus* ATCC 25923**	25	6.25	0.625 (A)	0.375 (S)	1.125 (I)	1.125 (I)	0.625 (A)	0.5 (A)
S. aureus	25	6.25	0.375 (S)	0.375 (S)	0.625 (A)	0.625 (A)	0.375 (S)	0.375 (S)
B. subtilis	50	25	0.625 (A)	0.25 (S)	0.375 (S)	0.25 (S)	0.375 (S)	0.25 (S)

KNO_2 – potassium nitrite, $NaNO_2$ – sodium nitrite
I – indifferent effect, A – additive effect, S – synergistic effect

The preservatives (potassium nitrite and sodium nitrite) showed different activity. Minimum inhibitory concentrations of sodium nitrite ranged from 6.25 mg/mL to 25 mg/mL, while potassium nitrite concentrations ranged from 25 mg/mL to 100 mg/mL (Tab. 2). Higher antibacterial activity was shown by sodium nitrite in relation to potassium nitrite. In the study

STANOJEVIĆ *et al*. (2009) sodium nitrate was active in the range of 0.5 - 2 mg/mL while UZOH *et al*. (2016) have expressed the activity of sodium nitrite as zones of bacterial growth inhibition and the diameters were 1 - 3 mm at 1% concentration. Compare the results, it can be noticed that the effectiveness of preservatives depends on type of bacterial strains.

The interaction between Curcuma longa extracts and preservatives

The results of combined acting of ethanol, ethyl acetate and acetone extract of *C. longa* and preservatives (potassium nitrite, sodium nitrite) expressed by FIC index are shown in Table 2. Synergistic, additive and indifferent effects were observed. FIC index was ranged in intervals from 0.25 to 2.0.

In relation to tested Gram-negative bacteria, indifferent effect was noticed. Exept, the combinations with sodium nitrite were additive for *E. coli* O157 and *P. mirabilis*. In this combination 1/8 MIC of extracts reduced MIC of sodium nitrate two times. The synergistic effect has occurred in relation to *S. aureus* ATCC 25923, *S. aureus* and *B. subtilis*. For strain *S. aureus* ATCC 25923 it was present only in the combination of ethanol extract and sodium nitrite. For *B. subtilis* and *S. aureus* both preservatives acted synergistically with plant extracts. These combinations decreased the MICs of preservatives 8-fold (Tab. 4). In case of mention strains of bacteria, MICs of preservatives tested alone were in range 50 – 6.25 mg/mL while in combination with plant extracts the MIC range was lower, 6.25 mg/mL – 1.56 mg/mL (Tab. 4).

The interaction between Urtica dioica extracts and preservatives

The results of combined acting of ethanol, ethyl acetate and acetone extract of *U. dioica* and preservatives (potassium nitrite, sodium nitrite) expressed by FIC index are shown in Table 3. Synergistic, additive and indifferent effects were observed. FIC index was ranged in intervals from 0.375 to 2.0.

Table 3. FIC index values showing different types of interaction between *Urtica dioica* extracts and preservatives.

Bacterial strains	**Ethanol extract**		**Acetone extract**		**Ethyl acetate extract**	
	KNO_2	**$NaNO_2$**	**KNO_2**	**$NaNO_2$**	**KNO_2**	**$NaNO_2$**
S. enterica	0.675 (A)	0.675 (A)	1.125 (I)	0.625 (A)	0.625 (A)	0.625 (A)
S. typhimurium	0.625 (A)	0.5 (S)	0.625 (A)	0.5 (S)	0.625 (A)	0.375 (S)
***E. coli* ATCC 25922**	0.75 (A)	1 (I)	1.125 (I)	1 (I)	1.125 (I)	0.625 (A)
E. coli	1 (I)	0.625 (A)	1.125 (I)	0.625 (A)	1.125 (I)	0.75 (A)
***E. coli* O157**	1.125 (I)	1.125 (I)	1.125 (I)	0.625 (A)	1.125 (I)	0.625 (A)
K. oxytoca	0.625 (A)	0.625 (A)	0.625 (A)	0.625 (A)	0.625 (A)	0.625 (A)
P. mirabilis	1.125 (I)	0.625 (A)	1.125 (I)	0.625 (A)	1.125 (I)	0.625 (A)
***S. aureus* ATCC 25923**	2 (I)	0.375 (S)	1.125 (I)	0.375 (S)	1.125 (I)	0.375 (S)
S. aureus	2 (I)	0.375 (S)	1.125 (I)	0.5 (S)	1.125 (I)	0.625 (A)
B. subtilis	1.125 (I)	0.625 (A)	1.125 (I)	0.625 (A)	1.125 (I)	1.125 (I)

KNO_2 – potassium nitrite, $NaNO_2$ – sodium nitrite
I – indifferent effect, A – additive effect, S – synergistic effect

The indifferent effect of extracts and potassium nitrite was observed against strains of *E. coli*, *E. coli* O157, *P. mirabilis*, *S. aureus* ATCC 25923, *S. aureus* and *B. subtilis*, while against these strains, the combination of plant extracts and sodium nitrite, in most cases, additive or synergistic effect was shown. The additive effect was noticed in relation to *K. oxytoca*, *S. enterica*, *P. mirabilis* and the MICs of sodium nitrite was reduced two times. Synergism was not observed in any combination of tested extracts and potassium nitrite. A synergistic effect was observed in the combination of all extracts and sodium nitrite in relation to *S. typhimurium* and *S. aureus* ATCC 25923, while against *S. aureus* species a synergistic effect was observed in the combination of ethanol and acetone extract. MICs of sodium nitrite in combination with 1/4 MIC or 1/8 MIC (2.5 – 0.625 mg/mL) of plant extracts were decreased up to 8-fold (Tab. 4).

Table 4. The synergistic effects of *Curcuma longa* and *Urtica dioica* extracts with preservatives

Curcuma longa						
Bacterial strains	**Ethanol extract**		**Acetone extract**		**Ethyl acetate extract**	
	KNO_2	**$NaNO_2$**	**KNO_2**	**$NaNO_2$**	**KNO_2**	**$NaNO_2$**
	MIC*					
***S. aureus* ATCC 25923**	/	$1/4_E + 1/8_P$	/	/	/	/
S. aureus	$1/4_E + 1/8_P$	$1/4_E + 1/8_P$	/	/	$1/4_E + 1/8_P$	$1/4_E + 1/8_P$
B. subtilis	/	$1/8_E + 1/8_P$	$1/4_E + 1/8_P$	$1/8_E + 1/8_P$	$1/4_E + 1/8_P$	$1/8_E + 1/8_P$
Urtica dioica						
S. typhimurium	/	$1/4_E + 1/4_P$	/	$1/4_E + 1/4_P$	/	$1/8_E + 1/8_P$
***S. aureus* ATCC 25923**	/	$1/4_E + 1/8_P$	/	$1/4_E + 1/8_P$	/	$1/4_E + 1/8_P$
S. aureus	/	$1/4_E + 1/8_P$	/	$1/4_E + 1/4_P$	/	/

"/" – no synergistic effect;
MIC* - the most active combination of extract ($_E$) and preservatives ($_P$)

Synergistic interaction between two agents, in which one agent enhances the effect of the other and together they act more efficiently than as individual agents represents a new approach to controlling pathogenic bacteria. Plants represent significant sources of antibacterial bioactive substances which possess an ability to enhance the activity of an antimicrobial agent in combination with it reducing its effective dose and decrease undesired side effects (STEFANOVIĆ, 2017). Based on the literature reviewed, there are no data on the studied interactions of ethanol, acetone, and ethyl acetate extracts of *C. longa* and *U. dioica* with potassium nitrite and sodium nitrite. However, other investigations have shown the synergism of plant extracts and preservatives and their join activity have decreased the effective doses of preservatives (STANOJEVIĆ *et al*., 2010a, b).

CONCLUSION

The results of this investigation confirm the potential efficacy of *Curcuma longa* and *Urtica dioica* ethanol, ethyl acetate, and acetone extracts in increasing the activity of preservatives and indicate their potential application as natural preservatives in order to protect and conserve food and thus reduce the doses of preservatives and negative side effects on human health. In synergistic combinations, it was observed that sub-inhibitory concentrations of extracts (1/4 MIC, 1/8 MIC) modified the activity of preservatives by reducing the effective concentrations up to 8-fold. Synergism was noticed against

Staphylococcus aureus ATCC 25923, *S. aureus*, *Bacillus subtilis* and *Salmonella typhimurium*. The ethanol extract and ethyl acetate extract of *C. longa* were the most promising agents for synergistic application with the preservatives. The significant amount of phenolic compounds contribute to the biological activity of these plants.

Acknowledgments

This investigation was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 41010).

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