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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Antimicrobial mechanism of the major active essential oil compounds and their structure–activity relationship

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Abstract Plant essential oils possess many sorts of bioactivities including microbicidal property. A number of essential oil components have been characterized as effective antibacterial agents. In this study, we selected several major antibacterial essential oil compounds and investigated their inhibition against 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the key enzyme of the 2-methyl-D-erythritol 4-phosphate terpenoid biosynthetic pathway and also a validated target for screening novel antibiotics. The results show that compounds eugenol and carvacrol display medium to weak inhibition against 1-deoxy-D-xylulose 5-phosphate reductoisomerase with IC_{50} values being about 97.3 and 139.2 μM, repectively; Compounds thymol, geraniol, linalool, and nerol exhibit weak 1-deoxy-D-xylulose 5-phosphate reductoisomerase inhibitory activity while perillaldehyde, cinnamaldehyde, α-terpineol, and citral possess undetectable inhibition against1-deoxy-D-xylulose 5-phosphate reductoisomerase. Based on these data, the structure–activity relationship of these compounds is discussed. Additionally, the inhibition kinetics of carvacrol and eugenol are also determinated. These results can not only deepen our understanding toward the antimicrobial mechanisms of eugenol and carvacrol, but also direct the reasonable application of these antimicrobial agents in medical pathology and in the control of plant diseases as well as in food industry.

Keywords 1-Deoxy-D-xylulose 5-phosphate reductoisomerase · Inhibition · Essential oils · Structure–activity relationship

Abbreviations

DLS	Dynamic light scattering
DXR	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
DXP	1-Deoxy-D-xylulose 5-phosphate
Eos	Essential oils
MEP	2-Methyl-D-erythritol 4-phosphate
NADPH	β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt

Introduction

It has long been known that plant essential oils (Eos) possess many sorts of bioactivities such as antimicrobial, antiviral, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties, among which the antimicrobial activity has been investigated thoroughly and well documented (Burt 2004; Hammer and Carson 2011; Friedman 2014). Recent studies in this area still largely concentrated on the determination of antimicrobial activities of various plant EOs against different microbials, such as human pathogenic bacteria (Lambert et al. 2001; Chang et al. 2001; Kim et al. 2003; Rossi et al. 2007; Chen and Zhong 2011) phytopathogenic bacteria (Lo Cantore et al. 2004, 2009;

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Iacobellis et al. 2005), foodborne pathogens (Faleiro et al. 2005; Catherine et al. 2012), food-spoilage yeasts (Sacchetti et al. 2005), phytopathogenic fungi (Pitarokili et al. 2008) etc., and their potential applications. A number of EO compounds such as carvacrol, thymol, eugenol, perillaldehyde, and cinnamaldehyde etc., which exhibit minimum bacteria inhibitory concentrations of 0.05–5 mg mL^{-1} in vitro, have been elucidated as effective antibacterial agents (Burt 2004; Friedman 2014). Meanwhile, some investigations centered on the mechanisms of bactericidal action of these active components of EOs (Lambert et al. 2001; Gill and Holley 2004, 2006; Di Pasqua et al. 2006; Devi et al. 2010; Lambert et al. 2001; Ultee et al. 2002).

DXR (1-Deoxy-D-xylulose 5-phosphate reductoisomerase), one of the key enzymes of the newly established MEP (2-methyl-D-erythritol 4-phosphate, **2**) terpenoid biosynthetic pathway (Rohmer 2010), catalyzes the first committed step of the alternative pathway, namely the conversion of DXP (1-deoxy-D-xylulose 5-phosphate, **1**, Fig. 1) to MEP in the presence of a divalent cation and NADPH (β-Nicotinamide adenine dinucleotide 2'-phosphate-reduced tetrasodium salt) through a retro-aldol–aldol mechanism (Wong and Cox 2007; Munos et al. 2009; Manning et al. 2012; Li et al. 2013). It is a promising target for the screening of novel antibiotics because the MEP pathway operates only in the human pathogens, such as *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Helicobacter pylori* and in the protozoan Plasmodium parasite (Pérezgil and Rodríguezconcepción 2013; Gräwert et al. 2011), but not in human beings, and disruption of the DXR step is lethal for the bacteria (Rohmer 2010). Many investigations have therefore been carried out to look for its inhibitors, which led to the discovery of fosmidomycin (**3**), a known component previously characterized from *Streptomyces lavendulae* and its congener FR900098 (**4**). Both of these two highly hydrophilic compounds not only possess strong inhibitory activity on DXR, but show potent antimicrobial activity as well (Jomaa et al. 1999). Clinical studies have indicated that **3** is somewhat effective in treating *Plasmodium falciparum*, the parasite that is responsible for malaria (Missinou et al. 2002). Up to now, more than 100 structural analogs of **3** have been prepared and their activities on DXR have been evaluated (Jackson and Dowd 2012). Several lipophilic compounds with structural similarity to **3** were recently synthesized and their DXR inhibitory effects were tested (Deng et al. 2009). Kaiser etc. have tried to seek DXR inhibitors from Mediterranean plants and found that the leaf extracts of *Cercis siliquastrum* exhibit strong inhibitory activity, but no specific compound has been elucidated (Kaiser et al. 2007). This represents the only research in this field, which was focused on the plant extracts.

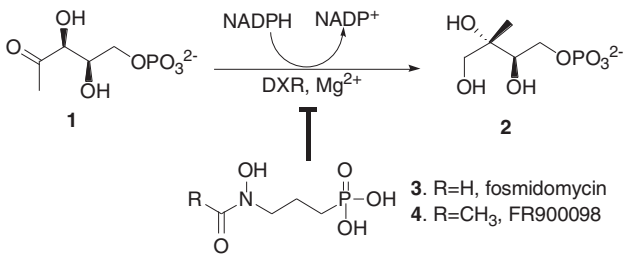

Fig. 1 The first committed step of the MEP terpenoid biosynthetic pathway and its inhibitors

In this paper, we report on functional bioassays to test the main effective components of plant EOs for inhibitory activity against DXR. In a pilot screen, we found that carvacrol and eugenol displayed medium to weak inhibition against DXR; Compounds thymol, geraniol, linalool, and nerol only exhibited weak DXR inhibitory activity while perillaldehyde, cinnamaldehyde, α-terpineol, and citral showed no inhibition against DXR (for the chemical structures of the EO compounds, see Supplementary Fig. S1 in the Supplementary Materials). Based on these results, we initially discussed the structure–activity relationship of these compounds. Moreover, we determined the DXR inhibition kinetics of carvacrol and eugenol as well. Herein we would like to disclose all the experiment details.

Materials and methods

Materials

Analytical grade carvacrol, thymol, and eugenol were purchased from Sigma-Aldrich (St. Louis, MO, USA); Cinnamaldehyde, α-terpineol, geraniol, linalool, citral, and nerol were the products of Alfa Aesar (Tianjin, China); Perillaldehyde was from Shanghai Yuanmu (Shanghai, China). The stock solutions of the EO compounds (10 mM) were prepared in distilled water containing 5% Tween-80 (W/V). NADPH was purchased from GEN-VIEW SCIENTIFIC INC. (Tallahassee, FL, USA); Fosmidomycin was from Toronto Research Chemicals Inc. (North York, Toronto, ON Canada); DXP was synthesized according to procedures previously published by this laboratory (Li et al. 2010). All other chemicals used were of analytical reagent grade.

Preparation of recombinant *Escherichia coli* DXR

The expression and purification of recombinant *E. coli* DXR were carried out in accordance with the reported procedure (Li et al. 2013).

Determination of the inhibitory activity of EO compounds against DXR using photometric assay

Assay mixtures comprised of 100 mM Tris-HCl, pH 7.4, 5.0 mM $MgCl_2$, 1.5 mM NADPH, 0.3% (W/V) Tween-80, and 6 μg/mL of DXR in a final volume of 120 μL. The EO compound (final conc. 150 μM) was added, and the mixture was then preincubated at 30 °C for 20 min before the addition of DXP (final conc. 1.8 mM) to start the reaction. In a control assay, fosmidomycin (**3**, final conc. 1.0 μM) was used instead of the EO compounds. The reaction mixtures were incubated at 30 °C for 30 min and the absorbance at 340 nm was subsequently recorded (Li et al. 2013).

Analysis of the particle size by DLS (Dynamic light scattering)

The particle size of carvacrol/eugenol in Tris-HCl buffter (100 mM, pH 7.4) containing 2% (V/V) DMSO was analyzed at room temperature in the absence and presence of DXR (end concentration: 6 μg/mL) on a DLS analyzer (NICOMP-380, Particle Sizing Systems Inc., Santa Barbara, Calif., USA). The detector time was 10 min. The concentrations of eugenol and carvacrol were at 0.2 and 0.3 mM, respectively. A solution of 2% (V/V) DMSO in 100 mM Tris-HCl buffter (pH 7.4) was referenced.

Docking experiment

Autodock 4.2.6 software was used for docking experiments, and the results were shown by Chimera 1.10.1 software. The profiles of the crystal of the DXR-NADPH-Mg^{2+}-fosmidomycin quaternary complex were obtained from Protein Data Bank (PDB accession code 2EGH). In the eugenol docking simulation, we removed fosmidomycin and used DXR-NADPH-Mg^{2+} ternary complex as a receptor and eugenol as a ligand. In the carvacrol docking simulation, fosmidomycin was removed, and subsequently DXP was docked into the binding sites of fosmidomycin because it had been suggested that DXP could be superposed exactly onto fosmidomycin (Sweeney et al. 2005). Then, the docking simulation was carried out using carvacrol as a ligand and the mimic DXR-NADPH-Mg^{2+}-DXP quaternary complex as a receptor.

Results

Photometric assay of DXR inhibitory activity of the EO compounds

Because the selected EO compounds carvacrol, thymol, eugenol, and cinnamaldehyde contain substituted aromatic ring that may produce absorbance at 340 nm at relatively high concentrations, they could interfere the photometric determination when added to the assay mixture. Therefore, we first tested the absorbance of all the selected compounds at around 0.6 mM at 340 nm. The results showed that the A_{340} of each compound was less than 0.02 at this concentration. So the maximum amounts of the EOs used in the assays were controlled under 0.6 mM. Meanwhile, we also measured the A_{340} of Tween-80 at 0.3% (W/V), which was employed to enhance the solubility of the EO compounds in aqueous medium. From the determination we found that 0.3% Tween-80 was acceptable for the screening procedure. We further checked the influence of Tween-80 on the activity of DXR at the same concentrations, and the result indicated that it did not retard the reaction. Based on the above measurements, 0.3% Tween-80 was chosen in the bioassay.

Taking the optimized method, we evaluated the DXR inhibitory activity of all the EO compounds at a final concentration of 150 μM, and the results revealed that at the selected concentration, compounds perillaldehyde, α-terpineol, citral, and cinnamaldehyde did not give any detectable effect against DXR, while geraniol and nerol exhibited very weak activity (10.61 and 5.19%). The data also showed that thymol and linalool only had weak activity against DXR (>20%, Table 1). Compounds eugenol and carvacrol displayed quite strong inhibition against DXR at this concentration. We further measured DXR inhibitory activities of eugenol, carvacrol, thymol, and linalool at different concentrations, and the results depicted in Fig. 2 showed

Table 1 DXR inhibitory activity of eugenol, carvacrol, thymol, and linalool at 150 μM, their IC_{50} values, and inhibition kinetics of eugenol, carvacrol

EO compounds	Inhibition (%)	IC_{50} (μM)[a]	Mode of inhibition	Ki (μM)
eugenol	81.63 ± 4.53	97.31 ± 2.75	Competitive	73.96 ± 4.81
carvacrol	58.92 ± 3.72	139.24 ± 3.49	Un-competitive	103.12 ± 5.16
thymol	28.53 ± 3.92	241.92 ± 3.93	ND[b]	ND
linalool	23.17 ± 3.86	273.11 ± 4.22	ND	ND
fosmidomycin[a]	100.21 ± 2.11	0.27 ± 0.011	ND	ND

[a] Fosmidomycin was used as a positive control at 1 μM, the reported IC_{50} for it against *E. coli* DXR is 0.37 μM (Kaiser et al. 2007)

[b] ND: not detected

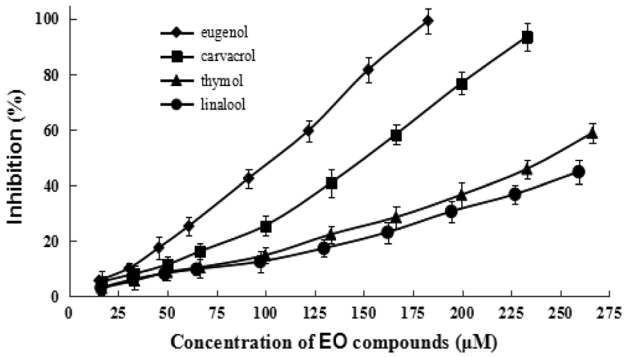

Fig. 2 Concentration-dependent inhibition of eugenol, carvacrol, thymol, and linalool against DXR

that all these compounds possessed clear concentration-dependent inhibitory modes against the target with IC_{50} values of about 97.3, 129.2, 241.9, 273.1 μM, respectively. Complete inhibition of DXR activity was observed when the concentration of eugenol reached 180 μM and carvacrol reached about 245 μM, respectively. In addition, compounds eugenol and carvacrol still exhibited approximately 10% inhibition against the protein at about 30 μM.

Analysis of the particle size by DLS

DLS is a widely used technique in material sciences to measure particle sizes in solutions (Seidler et al. 2003). Normally, the measurements of DLS produce two parameters, one is the scattering intensity, which depicts particle concentration; the other is average particle size calculated from the autocorrelation functions of the scattered laser light. In the current study, we employed this method to determine whether there were any particles appeared in the samples in which carvacrol or eugenol was diluted to an end concentration of around two times of its IC_{50} value in 100 mM Tris-HCl buffer (pH 7.4) in the absence or presence of DXR protein (6 μg/mL). From the data listed in Table 2, we can see that there is no detectable particle in all measurements. Therefore, in light of the criteria established by Shoichet and co-workers (Seidler et al. 2003), this observation implies the two compounds could inhibit DXR via a specific mechanism.

Determination of DXR inhibition kinetics of eugenol and carvacrol

To determine the modes of inhibition of carvacrol and eugenol against DXR, the initial enzyme kinetics was investigated over a fixed inhibitor concentration and at different DXP concentrations employing the photometric method described above. Lineweaver–Burk (LB) graphical charts were obtained via plotting the reciprocal of the reaction velocity against the reciprocal of the concentration of DXP. The results, as shown in Fig. 3a, disclosed that eugenol was a competitive inhibitor of DXR with a Ki of 73.9 μM. The mode of inhibition of carvacrol was found to be un-competitive (Fig. 3b) with a Ki value of 103.1 μM as listed in Table 1.

Table 2 Dynamic light scattering of carvacrol or eugenol plus DXR in 100 mM Tris-HCl buffer (pH 7.4)

	Conc (μM)[a]	Without DXR		With 6 μg/mL DXR	
		Count rate (KHz)	Size (nm)	Count rate (KHz)	Size (nm)
carvacrol	300	1.2 ± 0.3	N/A[c]	3.4 ± 0.5	N/A
eugenol	200	2.7 ± 0.7	N/A	3.8 ± 0.4	N/A
Blank[b]	–	3.4 ± 0.3	N/A	4.5 ± 0.4	N/A

[a] Final concentration of DMSO was 2% (V/V)

[b] Blank: 2% DMSO in 100 mM Tris-HCl buffter (pH 7.4)

[c] N/A: not available

The results of docking experiments

The results of docking experiments were shown in Supplementary Figs. S2 and S3 in the Supplementary Materials. As expected, both compounds bind at the active hydrophobic pocket of DXR. This result again implies that eugenol and carvacrol inhibit DXR probably due to their specific action on the active sites of DXR. The close-up views of the docking results depicted in Supplementary Fig. S3 display the interactions between the inhibitors and the cofactors/the residues. From A we can see that besides its coordination with the metal ion (green wire ball in the figure), eugenol binds to the enzyme through eight residues: Lys124, Asp149, Ser150, Trp211, Met213, Ile217, Asn226, and Glu230. It forms one H-bond (*purple bead wire*) with DXR (between its OH and the residue Asn226) and produces close contact with the other seven residues (*yellow wire balls*). In addition, it also has some close contact with NADPH (*blue wire balls*). Moreover, all the eight binding sites of eugenol are also the binding sites of DXP, the natural substrate of DXR (Deng et al. 2011; Reuter et al. 2005; Sweeney et al. 2005; Yajima et al. 2002, 2004). These data support that eugenol is a competitive inhibitor of DXR vs. DXP. From B we can observe that after DXP has combined with DXR, carvacrol can subsequently bind to the enzyme through generating close contact with DXP (black wire ball) and residues of DXR: Trp211, Ser212, Met213, Asp274, and Met275. In addition, carvacrol also forms an H-bond (*purple bead wires*) with DXR (between its OH and the residue Pro273). The five residues, especially Trp211 and Met213 are key to the activity of DXR

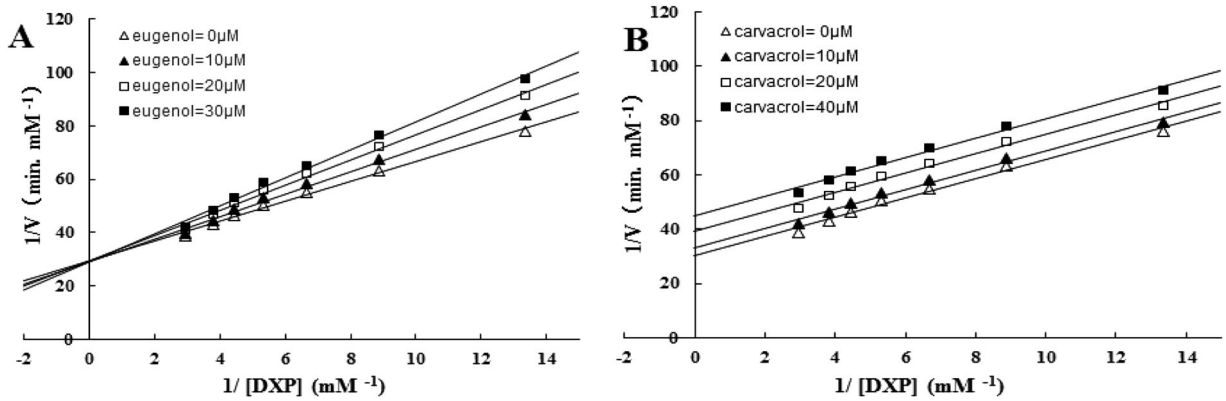

Fig. 3 LB plot of *E. coli* DXR in the absence and presence of eugenol (**a**) and carvacrol (**b**). **a** DXR inhibition kinetics of eugenol. Assay mixtures containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM NADPH, 0.3% (W/V) Tween-80, eugenol (0, 10, 20 or 30 μM), and 1 μg/mL of DXR in a final volume of 120 μL was preincubated at 30 °C for 5 min, then DXP (final conc. 0.033 to 0.18 mM) was added and the incubation was continued for another 20 min. **b**. DXR inhibition kinetics of carvacrol. Assay mixtures containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM NADPH, 0.3% (W/V) Tween-80, carvacrol (0, 10, 20 or 40 μM), and 1 μg/mL of DXR in a final volume of 120 μL was preincubated at 30 °C for 5 min, then DXP (final conc. 0.033 to 0.15 mM) was added and the incubation was continued for another 20 min

(Deng et al. 2011; Reuter et al. 2005; Sweeney et al. 2005; Yajima et al. 2002, 2004). In contrast to the binding mode of eugenol, carvacrol does not coordinate with Mg^{2+}. All above data support that carvacrol is an uncompetitive inhibitor of DXR vs. DXP.

DXR inhibitory activities of methylated eugenol, carvacrol, thymol and linalool

DXR inhibitory effects of the methylated EO compounds (for the methylation of these compounds see Supplementary Materials) were evaluated using the photometric assay. The data showed that methylated eugenol and carvacrol only displayed very weak DXR inhibitory activity (8.56 and 6.35%, respectively) at a concentration of approximately 150 μM and methylated thymol and linalool even completely lost their activity.

Discussion

Although the antimicrobial property of plant EOs was discovered more than half a century ago (Boyle 1955), it did not gain enough attention because normally the antimicrobial activities of plant EOs are medium or even weak. The recent enhancement of interest in "green" consumerism and the current threat due to the abuse of antibiotics and pesticides have resulted in a renewal of scientific interest in these materials, because generally to say, the plant EOs as antimicrobial agents not only mean more safety to human beings and more friendly to the environment owing to their natural origin, but also represent low risk for drug-resistance development by pathogenic microbials. The reasonable application of EOs and/or their constituents in medical pathology and in the control of plant diseases as well as in food industry to inhibit the microbials malgenic to consumers and/or to be responsible for food spoilage has been investigated by several studies (Burt 2004; Lo Cantore et al. 2009; Daferera et al. 2003; Wolf et al. 2008; Tinivella et al. 2009). However, the exploration of the mechanistic actions of the plant EOs and/or their main components still stays at its early stage (Lambert et al. 2001; Gill and Holley 2004, 2006; Di Pasqua et al. 2006; Devi et al. 2010; Lambert et al. 2001; Ultee et al. 2002). Therefore, we chose 10 EO compounds whose antimicrobial activities have been proven to test whether they have inhibitory effect against DXR, and by doing this, we might not only find some lipophilic inhibitors of DXR from the EO compounds, but also elucidate their mode of action as well.

Among the ten EO compounds selected, the aromatic carvacrol, thymol, eugenol, and cinnamaldehyde appear to have received the most attention from investigators because of their excellent antimicrobial activities and they are thought to be the main effective components of plant EOs. The six aliphatic components, namely nerol, linalool, citral, geraniol, perillaldehyde, and α-terpineol, possess antimicrobial effects that are comparable to the four aromatics (Burt 2004). It is generally regarded that the antimicrobial mechanism of these EO compounds is that their lipophilicity enables them to partition in the lipophilic lipids of the cytoplasmic membrane and mitochondria, disturbing the structures, causing them to be more permeable and resulting in leakage of cell contents (Burt 2004; Hammer and Carson 2011; Di Pasqua et al. 2006; Gill and Holley 2006; Devi et al. 2010). Studies also showed that the phenolic hydroxyl groups of carvacrol, thymol, and eugenol, and the presence

of a delocalized electrons system are important for their antimicrobial effect because (i) methylation of these aromatic compounds caused loss of their activity; (ii) menthol which is an aliphatic analog of carvacrol/thymol, lacks activity (Ultee et al. 2002; Griffin et al. 1999; Knoblock et al. 1989).

Our determination discloses the structural prerequisites for DXR inhibitory activity of these EO compounds, that is the presence of a delocalized electrons system containing a hydroxyl group because (i) neither the three aldehydes (cinnamaldehyde, perillaldehyde, and citral), either aromatic or aliphatic, nor α-terpineol exhibits detectable inhibition against DXR; (ii) the three acyclic compounds geraniol, nerol, and linalool, that have an allylic alcohol moiety, show weak to medium effect against the protein. It seems that terminal double bond allylic alcohol (e.g. linalool) is better than non-terminal double bond allylic alcohol (e.g. geraniol and nerol); (iii) the phenolic compounds eugenol and carvacrol display best activity against DXR. Further analysis reveals that the DXR inhibition activity of these EO compounds relates to the size of their delocalized electrons system. Bigger system is beneficial for the activity, for example eugenol and carvacrol are better than the three allylic alcohols and eugenol is better than carvacrol. An exception is thymol who only exhibits an activity half of that of its isomer carvacrol, which could be because the OH of thymol is in ortho-position of the bulky isopropyl that could hindrance the effective interaction of the compound with the target. This is different from the observation that carvacrol and thymol possess comparable antibacterial activity (Lambert et al. 2001; Ultee et al. 2002). This might be because that on the intact cell level, the intensity of the two compounds is dominated by their lipophilicity, thus the relative position of the OH group on the aromatic ring does not show strong influence, but on the enzyme level, the activity of them is based mainly on the interaction between DXR and the phenolic OH. The impact of the OH group on DXR inhibition activity is further confirmed by derivation experiment because the methylated eugenol, carvacrol, thymol, and linalool exhibited only weak or even no DXR inhibitory activity. It would be plausible that these EO compounds combine the OH group to DXR and decrease the activity of the protein. Early studies also pointed out the alkenyl substituting group(s) of non-phenolic EO compounds would positively influence their antibacterial activity (Dorman and Deans 2000), and this might partially explain why eugenol is a more potent inhibitor of DXR than carvacrol (Table 1).

Furthermore, our data show that eugenol and carvacrol cannot induce aggregation of DXR at concentrations about two times of their IC_{50}, indicating that they can specifically inhibit the activity of DXR. These observations are supported by the docking experiments. Some lipophilic phenolic compounds were prepared and found to be able to specifically suppress the activity of DXR with their IC_{50} values being in the same order of magnitude as the IC_{50} of eugenol and carvacrol (Deng et al. 2009). Although it has been deduced that putative hydrophobic inhibitors often might be promiscuous, non-specific inhibitors of DXR (Zingle et al. 2014), eugenol, carvacrol, and those synthetic compounds could be exceptional to this deduction. Our experiments also show that DXR inhibition kinetics of eugenol and carvacrol are completely different, (Table 1) although they have quite similar chemical scaffold. This result, which is supported well by the docking experiments, reflects that the interaction between eugenol and DXR is totally distinct from that between carvacrol and DXR, which deserves in-depth investigation.

Conclusion

In this paper, the DXR inhibitory effect of ten EO compounds that possess antimicrobial activity was tested and their structure–activity relationship was initially discussed. Combining the previous results (Burt 2004; Hammer and Carson 2011; Friedman 2014) and our observation, we would conclude that the EO components such as, eugenol and carvacrol et al. could be multi-targeted antimicrobial agents with a cascade mode of action. The primary mechanism of these compounds would be disruption of the cell membrane through binding with the lipophilic parts of the membrane. The secondary mechanism could be a specific inhibition of the function of DXR. Whether they target other protein(s) still needs further investigation. The results obtained in this study could be very useful to elucidate the mode of action of these EO compounds in one hand; they would be of importance in directing the reasonable use of these components in food industry and in agriculture in the other.

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Compliance with Ethical Standards

Competing interests The authors declare no competing financial interests.

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