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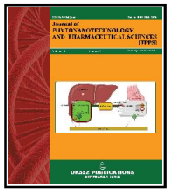

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Combined *in vitro* and *in vivo* antibacterial effect of catechin and linalool in rats

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Abstract

In vitro, combined antibacterial effect of catechin and linalool was evaluated by determining fractional inhibitory concentration index (FICI) against various Gram-positive and negative typed cultures and found additive effect against *Escherichia coli*, *Proteus mirabilis*, *Bacillus subtilis* and *Streptococcus pyogenes*, whereas, antagonistic effect against *Staphylococcus aureus*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*. Using a neutropenic rat thigh infection model, the *in vivo* combined antibacterial activity of catechin and linalool was assessed and demonstrated a strong antibacterial impact that was comparable to that of catechin and linalool alone. The results of the current experiment encourage further research on phytochemical combinations as well as the combination of phytochemicals with conventionally used antibacterial drugs to reduce the dose of synthetic antibacterial drugs and thereby to reduce antibacterial resistance and side effects.

1. Introduction

Many pathogens are now getting resistance against various antibacterial drugs and very limited new molecules are approved. Some antimicrobials are kept reserved for complicated clinical cases and hospital use. In this scenario, new alternatives, *viz.*, natural compounds should be tried for the treatment of pathogenic diseases (Parveen *et al*., 2020). In Ayurveda, polyherbal formulations are used effectively rather than single molecules. Amongst all phytochemicals present in plants, flavonoids and terpenoids show major pharmacological effects, including antibacterial, antioxidant, anticancer, anti-inflammatory, and antiprotozoal properties (Tapas *et al*., 2008; Ferreyra *et al*., 2012; Malik *et al*., 2020).

Catechin is a flavonoid and is derived from the extract of *Acacia catechu* L. (Tsuchiya, 2001). Catechins are distributed in a variety of foods and herbs and are widely studied for their pharmacological effects (Bansal *et al*., 2013; Grzesik *et al*., 2018; Akinmoladun *et al*., 2018). Linalool is an acyclic monoterpene tertiary alcohol and is derived from plants mainly Lamiaceae, Lauraceae and Rutaceae families (Aprotosoaie *et al*., 2014). Linalool is known to reveal various pharmacological activities such as antimicrobial, anti-inflammatory, antioxidant and anticancer properties (Kamatou and Viljoen, 2008). Looking at the great therapeutic potential of catechin and linalool, this study was planned to evaluate the combined *in vitro* and *in vivo* antibacterial efficacy of catechin and linalool in rats following intramuscular administration.

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2. Materials and Methods

2.1 Animals and ethical statement

The study was conducted in female albino wistar rats (n=18) weighing 353 ± 4.81 g to evaluate *in vivo* antibacterial activity. The experimental protocols were approved by Institutional Animal Ethics Committee of Veterinary College, Navsari, Gujarat.

2.2 Chemicals and reagents

Catechin hydrate (>98%), linalool (97%) and iodonitrotetrazolium chloride (INT) were purchased from Sigma-Aldrich, St. Louis, USA. Indomethacin was obtained from Calbiochem. Triethanolamine was purchased from MP biomedicals, USA. Dimethylsulfoxide (DMSO) and PEG-200 were purchased from Merck Specialties Private Limited, Mumbai. Chloramphenicol was procured from Himedia Laboratories Private Limited, Mumbai. Typed bacterial cultures were procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. HPLC-grade de-ionized water was used in all experimental procedures.

2.3 *In vitro* combined antibacterial effect of catechin and linalool

Minimum inhibitory concentrations (MICs) of catechin and linalool were determined individually for different Gram-positive organisms like *Staphylococcus aureus* (ATCC25923), *Streptococcus pyogenus* (ATCC8668), *Bacillus subtillis* (ATCC9372) and Gram-negative organisms like *Escherichia coli* (ATCC25922), *Salmonella typhimurium* (ATCC23564), *Pseudomonas aerugonosa* (ATCC27853) and *Proteus mirabilis* (NCIM2241) by microbroth dilution technique (Wiegand *et al*., 2008). Fractional Inhibitory Concentration (FIC) in combination with each other was also determined against above said organisms using a chequerboard assay (Odds, 2003; Duarte *et al*., 2012). FIC and FICI were calculated for each well in the plate using equation: Fractional Inhibitory

Concentration Index (FICI) = FIC_A + FIC_B. Where, FIC_A = (Concentration of drug A in well/MIC of individual drug A) and FIC_B = (Concentration of drug B in well/MIC of individual drug B). The lowest FICI value for each combination against each bacterium was taken into consideration and interpreted. If, the FICI value was less than or equal to 0.5, then synergistic, between 0.5 and 4.0, then additive or no interaction and if, the FICI value was more than 4.0, then the antagonist effect was considered for that combination against the studied organism. Catechin stock (40 mg/ml) was prepared using triethanolamine: DMSO: water in 0.5:0.5:9.0 ratio. Chloramphenicol (250 µg/ml) was used as a positive control after dissolving in sterile water. All bacterial cultures were prepared to Mcfarland 0.5 standard equivalents to 1.5×10^8 cfu/ml and final dispensing concentrations were made as 1.5×10^6 cfu/ml diluted with sterile broth. All microtiter plates were incubated and then all wells were supplemented with 30 µl freshly prepared iodonitrotetrazolium chloride (INT) dye (1 mg/ml) for evaluation of visual viability of organisms. This assay was performed in triplicate.

2.4 *In vivo* combined antibacterial effect of catechin and linalool in rat

In vivo, the antibacterial efficacy of catechinand linalool was evaluated in a neutropenic rat thigh infection model (Zhao *et al*., 2016). A total of eighteen rats were divided into three groups with six rats in each group. Catechin was dissolved using DMSO: PEG-200: 1-Methyl-2 pyrrolidone in a 4.5:4.5:1.0 ratioand linalool was diluted with DMSO for intramuscular administration in rats. Group I animals were treated with only bacterial suspension (0.2 ml, IM) (growth control). Group II animals were treated with bacterial suspension (0.2 ml, IM in thigh) and vehicle (vehicle control). Group III animals were treated with bacterial suspension (0.2 ml, IM) and a combination of catechin (200 mg/kg IM) and linalool (100 mg/kg IM). The neutropenic rat model was prepared by intraperitoneal administration of cyclophosphamide on day 1 (150 mg/kg) and day 4 (100 mg/kg). After confirmation of neutropenic condition, rats were infected with 0.2 ml bacterial suspension of *E. coli* ATCC25922 (1.5×10^8cfu/ml) in the left thigh on the same day. Drugs and vehicles were administered intramuscularly at 2 h and 8 h post-infection in the right thigh. One gram samples of the infected site's thigh muscle were taken after euthanasia in a sterile environment, after a twenty-four-hour period. On EMB agar plates, suitable dilutions of samples were streaked. The plates were then incubated overnight at 37°C, and bacterial colonies were counted using a colony counter.

3. Results

3.1 *In vitro*combined antibacterial effect

A combination of catechin and linalool was found to possess an additive effect with FIC index values 2.0, 2.1, 4.0 and 4.0 against *E. coli, S. pyogenus, P. mirabilis* and *B. subtillis*, respectively, whereas, antagonistic effect with FICI values 8.0, 8.0 and 16.0 against *S. aureus, S. typhimurium* and *P. aerugonosa*, respectively. MIC values of individual drugs and in the presence of another drug including FIC values against all studied organisms are given in Table 1. Representative photographs of *in vitro* chequerboard assay for FICI (catechin + linalool) against *E. coli* are depicted in Figure 1.

Table 1: Fractional inhibitory concentration index (FICI) and effects for the combination of catechinand linalool

Organism	Drug	MIC (mg/ml)		FIC	FICI	Effect
		Alone	Combination			
Staphylococcus aureus ATCC25923	Catechin	5.00	0.08	0.02	8.0	Antagonistic
	Linalool	1.25	10.00	8.00		
Escherichia coli ATCC25922	Catechin	10.00	0.08	0.01	2.0	Additive
	Linalool	0.63	1.25	1.98		
Salmonella typhimurium ATCC23564	Catechin	10.00	0.08	0.01	8.0	Antagonistic
	Linalool	1.25	10.00	8.00		
Pseudomonas aeruginosa ATCC27853	Catechin	5.00	0.63	0.13	16.0	Antagonistic
	Linalool	0.63	10.00	15.87		
Proteus mirabilis NCIM2241	Catechin	5.00	0.08	0.02	4.0	Additive
	Linalool	1.25	5.00	4.00		
Bacillus subtilis ATCC9372	Catechin	5.00	0.08	0.02	4.0	Additive
	Linalool	1.25	5.00	4.00		
Streptococcus pyogenes ATCC8668	Catechin	1.25	2.50	2.00	2.1	Additive
	Linalool	1.25	0.16	0.13		

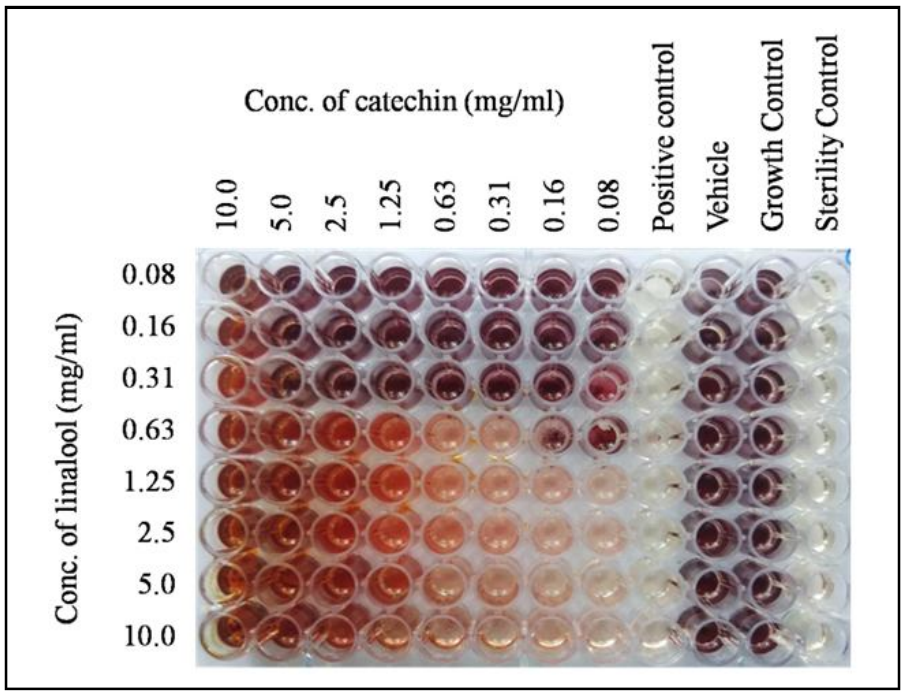

Figure 1: ***In vitro*** **chequerboard assay for FICI (catechin + linalool) against** ***E. coli.***

3.2 *In vivo* combined antibacterial effect

E. coli colony counts were carried out and converted into $\log_{10}$cfu/ml for the combination of catechin and linalool (Table 2 and Figure 2). Combination of linalool and catechin was found to have statistically significant antibacterial properties compared to growth and vehicle control against *E. coli* ($1.5x10^8$cfu/ml) in a neutropenic thigh infection model. Comparison of the bacterial colony count between groups of test drugs including control groups are shown in Figure 3.

Table 2: $\log_{10}$cfu/ml of *E. coli* ($1.5x10^8$cfu/ml) in infected thigh samples of rats treated with drugs including control groups (n=6)

Treatment groups	Bacterial colony count ($\log_{10}$cfu/ml)						Mean ± S.E.
	Rat number						
	R1	R2	R3	R4	R5	R6	
Growth control	5.40	5.28	5.44	5.20	5.32	5.42	5.35 ± 0.03^a
Vehicle control	5.44	5.23	5.39	5.28	5.36	5.33	5.34 ± 0.03^a
Catechin + Linalool	4.34	4.23	4.18	4.15	4.26	4.40	4.27 ± 0.04^b

Means bearing different superscripts between treatment groups differ significantly ($p<0.01$).

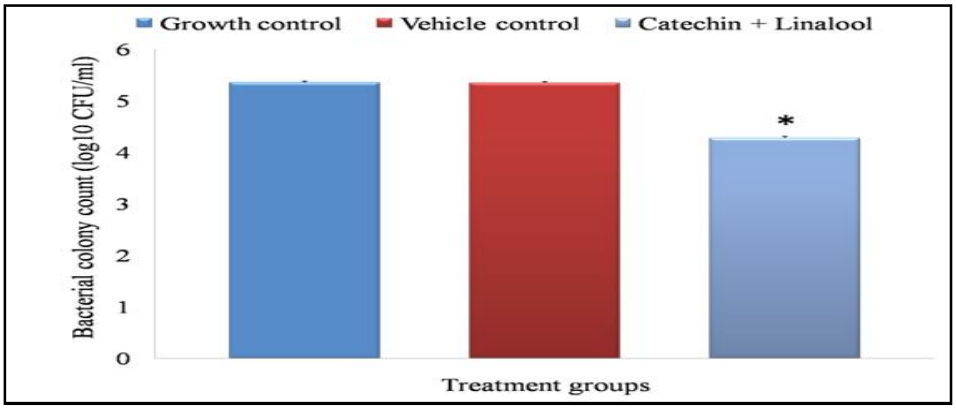

Figure 2: **$\log_{10}$cfu/ml of *E. coli* (1.5×10^8cfu/ml) in infected thigh samples of rats treated with drugs including control groups ($p<0.01$).**

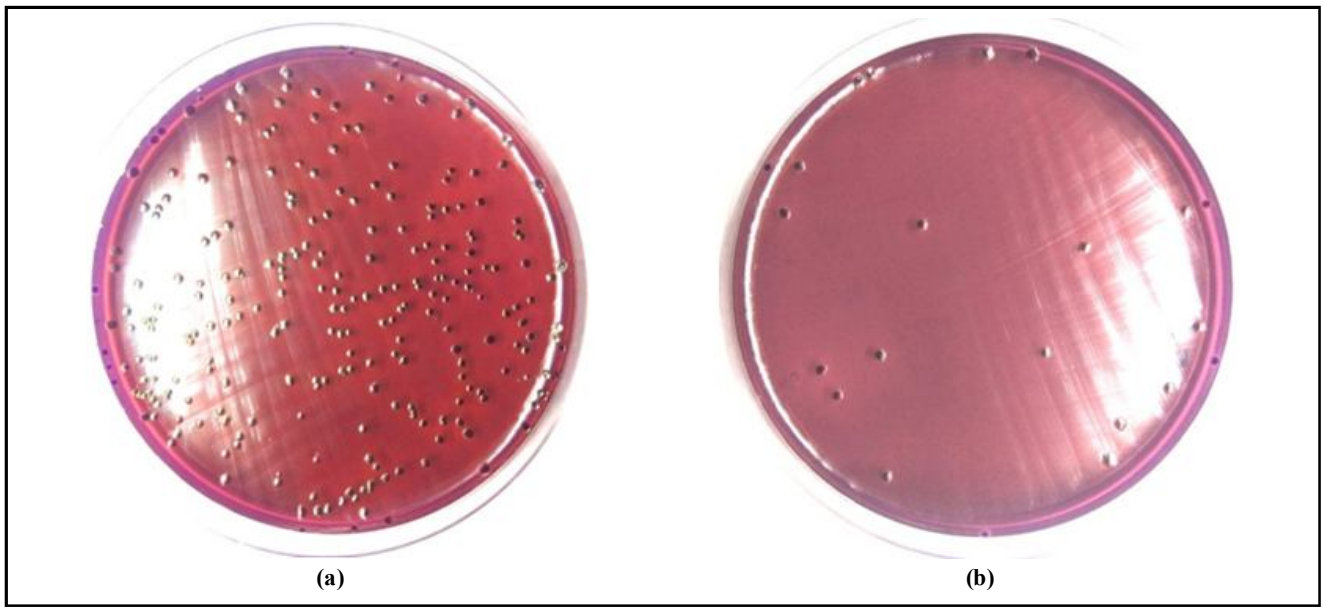

Figure 3: Representative photographs of EMB agar plates after *in vivo* antibacterial assay: (a) growth control; (b) combination of catechin and linalool.

4. Discussion

In the present study, the catechin and linalool combination showed an additive effect against some bacteria. Linalool in the present study is the constituent of various essential oil and earlier studies also noted a synergistic antibacterial effect against resistant organisms (Rakholiya *et al*., 2013; Iseppi *et al*., 2021; Bassole *et al*., 2010; Pei *et al*., 2009). Catechin is a flavonoid and earlier studies on combination including catechins showed synergistic antibacterial and antioxidant activities (Bernal-Mercado *et al*., 2018; Diaz-Gomez *et al*., 2014).The mixture of phenolic compounds was more effective in preventing cell adhesion and eradicating pre-formed biofilms of uropathogenic *E. coli* than single compounds and nitrofurantoin, and showed antioxidant synergy (Bernal-Mercado *et al*., 2018). In addition, the present studied combination was found ineffective against *S. aureus, S. typhimurium* and *P. aerugonosa* whichis in line with an earlier study done by Hartini *et al*. (2018). They found antagonistic antibacterial effect of the betel and red betel combination which comprises various flavonoids and terpenes against *S. aureus*, *S. epidermidis* and *E. coli*. Higher MICs of linalool were determined when co-administered with catechin compared to given alone, against all studied organisms. Possible mechanisms for antagonistic effect in combination may be due to chemical inactivation interaction and due to decreased penetration of compounds in organisms or increased efflux of drug molecules from bacteria. The combination of catechinand linalool in the present experiment showed *in vivo* antibacterial effect in neutropenic rat thigh infection model with *E. coli* load as 4.27 ± 0.04 $\log_{10}$cfu/ml which was significantly lower compared to growth control. This finding is supported by the additive antibacterial effect against *E. coli* observed for *in vitro* chequerboard assay in the present study.

5. Conclusion

In vitro combination antibacterial effect of catechin and linalool showed an additive effect against *S. pyogenus, B. subtillis, E. coli* and *P. mirabilis* whereas, an antagonistic effect against *S. aureus, S. typhimurium* and *P. aeruginosa*. Catechin and linalool in combination exhibited significant *in vivo* antibacterial activity in neutropenic thigh infection (*E. coli*) model in rats when administered intramuscularly. Research to date indicates that individual phytochemicals may not be effective but a combination has a significant antibacterial effect and that is the reason for the increased use of polyherbal formulations instead of single herb. Further research should be done to develop injected herbal formulations to increase bioavailability that helps to combat antibacterial drug resistance.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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