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Radical scavenging and antibacterial activity of caffemides against gram positive, gram negative and clinical drug resistance bacteria

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ABSTRACT

A new series of caffemide were synthesized and their antioxidant and antibacterial activities were explored. Antioxidant and antibacterial activities were measured of different structures of caffemide containing different functional groups. Anti-oxidative caffemides **1b** and **1g** showed significantly higher activity against different bacteria with MIC values less than 50 μg/ml. These anti-oxidative and antibacterial properties of caffemides might be helpful for the treatment of secondary infections and discovery of new antibiotics.

Caffeic acid and its derivatives widely distributed in fruits, vegetables, grains, coffee, barks and roots have dietary significances.[1] Caffeic acid containing catecholic moiety has anti-inflammatory, anti-cancer, antibacterial, antiviral and variety of pharmacological effects.[2] Density functional theory calculations reveal that the presence of at least two adjacent hydroxy groups (caffeic acid) on a phenyl ring containing electron withdrawing moiety have relatively weak bond dissociation energy of the phenolic–OH bond. It enables the production of phenoxy radicals which are stabilized by delocalization of their unpaired electron through extended conjugation and acting as potent antioxidant.[3] Over the last decades, many biological activities of ester and amide derivatives of caffeic acid have been explored both *in vivo* and *in vitro* study by many research groups. It was reported that the ester linkage of caffeic acid ester was metabolically labile and the half life of such ester was lower in presence of hydrolytic enzyme.[4] Caffeic acid amide (caffemide) is popular antioxidant agent and has many bioactive properties due to their structural stability both *in vivo* and *in vitro*.[4,5] Amide derivatives of caffeic acid have many pharmacological activities such as antioxidant, anti-hyperglycemic, anti-platelet, inhibitory effects on PG synthetase, MMP-2, MMP-9 and arachidonate 5-lipoxygenase.[6] In 2012 Jia et al. published that Danshensue-cysteine analog connected with caffeic acid by sulphur linkage had protective activity on cardiovascular system.[7] Moreover, caffemide had antiviral effects against influenza virus and showed inhibitory activities on neuraminidases with MIC value 7.2 μM.[8] The docking analysis indicated that the 3,4-dihydroxyphenyl group of caffemide could penetrate deeply into the active site of neuraminidases and showed hydrogen bonding interactions with Glu119 and Arg156 residues.[8] Recently, Hai-liang Zhu et al. reported that, a series of caffemides had considerable antibacterial activities against *B. subtilis*.[9] Some hydroxycinnamic acid amides and its analogues were synthesized as well as studied against *S. aureus* and HIV-1 integrase inhibitors.[10] Moreover, reactive oxygen species alter cellular and molecular signaling which results pathogenesis in gut mucosa of gastrointestinal tract.[11]

The presence and position of different functional groups responsible for antioxidant as well as antibacterial activities of caffemides have discussed in this paper. It would be helpful for inexpensive synthesis of novel caffemides having antioxidant and antibacterial properties.

Caffemides were synthesized by coupling reaction where caffeic acid and different substituted amines were taken as starting materials in dry tetrahydrofuran (THF). *N*,*N*′-Dicyclohexyl carbodiimide (DCC) was added as coupling reagent (Scheme 1).

The synthesized amides **1a** to **1m** were tested for radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.[12] From all UV spectra, the percentage of inhibition with various concentrations of test samples was calculated using following equation

$$\% \text{ Inhibition} = [\{(A\ control - A\ test)/A\ control\} \times 100]$$

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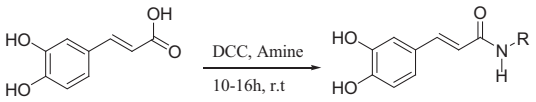

Scheme 1.

where *A control* was absorbance of the control (DPPH without test sample) and *A test* was absorbance of DPPH solution with caffemides. The radical scavenging activity of caffemides was expressed in terms of EC_{50} which defined as concentration of μM required for 50% decreased in absorbance of DPPH radical at 517 nm. A plot% of inhibition against concentration was drawn to establish the standard curve and calculated EC_{50} value of synthesized caffemides (Fig. 1).From Fig. 1, the observed EC_{50} values of caffemides were presented in tabular form (Table 1).

It was observed from Table 1, electron withdrawing group attached with caffemide had lower EC_{50} value than caffeic acid i.e. they behaved more potent antioxidant with respect to caffeic acid whereas electron donating moiety attached with caffemide had higher EC_{50} value compared to caffeic acid.

In vitro antibacterial activity of synthesized caffemides was done against a panel of human pathogens belonging to Gram-positive (*S. aureus*), Gram negative (*P. aeruginosa*), acid fast (*M. smegmatis*) and multidrug *S. aureus* MRSA with different time interval by colony forming unit (CFU assay).[13] Among all of the tasted caffemides (**1a–1m**), compounds **1b** and **1g** were exhibited significant antibacterial activity against tested microorganisms in a dose-dependent manner. Caffemides which had minimum inhibitory concentration (MIC) value greater than 50 μg/ml were not considered in this study. Exposure to 50 μg/ml of compound **1b** was found to kill 63.8% and 45.6% of the *M. smegmatis* population,

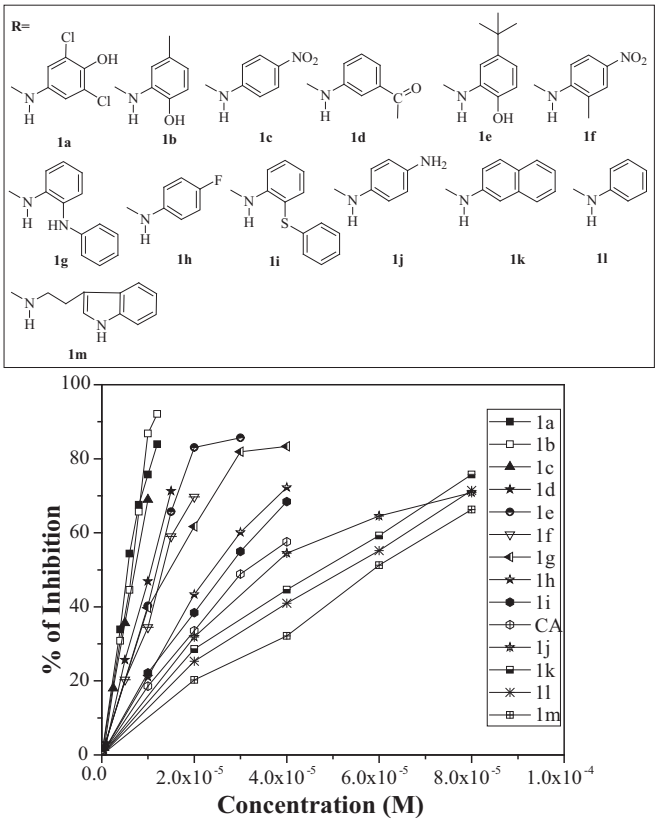

Fig. 1. % of inhibition of synthesized caffemides. Final concentration of DPPH was 100 μM.

Table 1
EC_{50} values of caffemides.

Compound	EC_{50} ± S.D (μM)	Compound	EC_{50} ± S.D (μM)
1a	5.51 ± 0.9	**1h**	24.06 ± 1.42
1b	6.57 ± 1.12	**1i**	27.04 ± 1.39
1c	7.21 ± 1.64	**CA**	30.88 ± 1.09
1d	10.62 ± 1.71	**1j**	36.01 ± 2.28
1e	11.90 ± 1.38	**1k**	47.30 ± 2.15
1f	13.18 ± 1.75	**1l**	52.63 ± 1.55
1g	14.67 ± 1.38	**1m**	58.81 ± 2.32

Data are shown as μM ± S.D of three independent trials. The final concentration of DPPH ethanolic solution was 100 μM.

whereas exposure to 50 μg/ml of compound **1g** was found to kill 51.9% and 78.1% of the *M. smegmatis* population after 6 h and 24 h respectively (Fig. 2a and b). Compounds **1b** and **1g** could completely eliminate *M. smegmatis* colonies beyond concentration 200 μg/ml after 6 h and 24 h treatment periods. In case of Gram-negative bacteria, approximately 50% of *P. aeruginosa* population was killed between 10–50 μg/ml and 50–100 μg/ml by compound **1b** after 1 h and 3 h respectively (Fig. 2c). But exposure to 50 μg/ml of compound **1g** was found to kill 58.9% and 62.1% of the *P. aeruginosa* population after 1 h and 3 h incubation period (Fig. 2d). It was found that both **1b** and **1g** could completely eliminate *P. aeruginosa* colonies beyond the concentration 300 μg/ml after 1 h and 3 h treatment period.

Among all tested bacteria, *S. aureus* WT was found to be more susceptible to compound **1b** such that 60.9% of bacterial population was killed between 1 and 10 μg/ml after 6 h incubation period. Treatment with doses 200 μg/ml and 50 μg/ml of compound **1b** was killed approximately 93.8% and 89.6% *S. aureus* WT bacterial colonies after 6 h and 24 h exposure period respectively (Fig. 3a). In case of compound **1g**, 86.8% and 68.2% of bacterial population was killed with dose 50 μg/ml against *S. aureus* WT after 6 h and 24 h treatment period. Moreover, compound **1g** with dose 100 μg/ml, 92.3% and 84.9% bacterial colonies were eliminated within 6 h and 24 h respectively (Fig. 3b). It was also observed that caffemides **1b** and **1g** were effective against *S. aureus* drug resistant bacteria and were found to kill 57.5% and 52.8% of *S. aureus* MRSA at concentration 50 μg/ml after 6 h, whereas exposure to 50 μg/ml of compound **1b** and **1g** were found to kill 64.8% and 61.2% of the *S. aureus* MRSA population after 24 h incubation period (Fig. 3c and d). No viable colonies were found beyond the concentration 100 μg/ml by compounds **1b** and **1g** after 6 h and 24 h treatment period.

This experimental study was designed in three different ways to understand the role of functional groups of caffemides were responsible for antioxidant and antibacterial properties. Ring A was fixed but the ring B contained electron donating, electron withdrawing and hydroxyl group present along with electron donating and withdrawing moiety (Fig. 4). Electron withdrawing group attached with caffemides (**1a**, **1c**, **1d** and **1f**) had lower EC_{50} values compared to caffeic acid (30.88 μM) and vitamin E (50.85 μM) i.e. they exhibited more potent antioxidant whereas electron donating moiety attached with caffemides (**1j–1m**) showed less antioxidant property with respect to caffeic acid. Compounds **1b** (R^1 = OH, R^4 = CH_3) and **1e** (R^1 = OH, R^4 = *t*-Bu) showed less EC_{50} values due to presence of an extra hydroxyl group (R^1 = OH) at ring B which probably took part to scavenge the free radical (Fig. 4). Compound **1a** (R^3 = OH, R^2 and R^4 = Cl) containing electron withdrawing moiety along with hydroxyl group (R^3 = OH) in ring B displayed lowest EC_{50} value.

The observed EC_{50} value of caffemide **1g** (R^1 = NH-Ph) was lesser than **1i** (R^1 = S-Ph) and lesser than caffeic acid. Compounds having radical scavenging activity would help to reduce the unnecessary stress inside the cell. It was well accepted that various

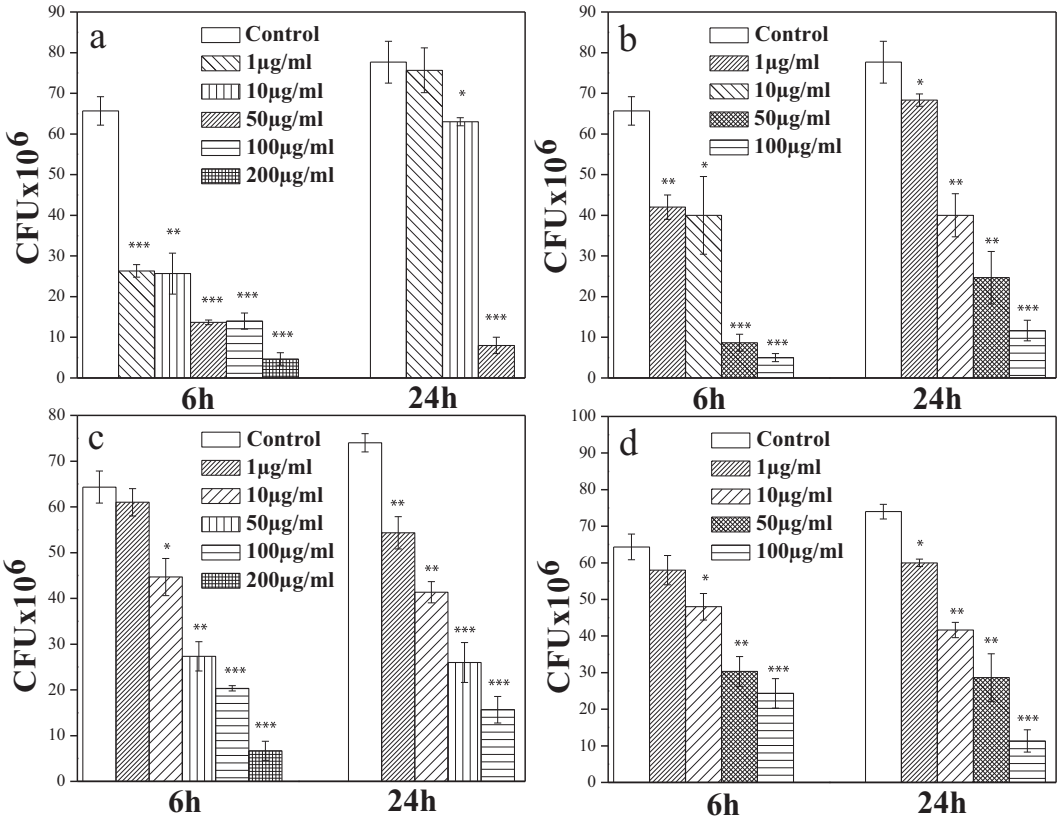

Fig. 3. (a) Activity of **1b** against *Staphylococcus aureus* WT. (b) Activity of **1g** against *Staphylococcus aureus* WT. (c) activity of **1b** against *Staphylococcus aureus* MRSA. (d) Activity of **1g** against *Staphylococcus aureus* MRSA. Values were the means ± S.D of three independent trials. The results were found to be statistically significant $p \leqslant 0.05$ (∗), $p \leqslant 0.001$ (∗∗), $p \leqslant 0.0001$ (∗∗∗) compared to that of control group without treatment by **1b** and **1g**.

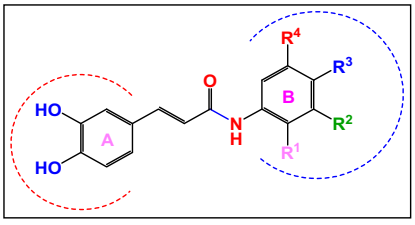

Fig. 4. Schematic representation of functional groups on caffemides.

bacteria could attack the cell in higher oxidative stress conditions. On the basis of antioxidant property, antibacterial activities of caffemides were investigated. The experimental result revealed that, caffemides **1c** ($R^3 = NO_2$) and **1d** ($R^2 = COCH_3$) had more radical scavenging property but showed toxic effect towards tested bacterial colonies due to presence of strong electron withdrawing group (Fig. 4). Caffemides **1a**, which had highest antioxidant activity was also toxic at a dose 1 μg/ml. However, compounds **1f** ($R^1 = CH_3$, $R^3 = NO_2$), **1h** ($R^3 = F$) and **1i** ($R^1 = S\text{-}Ph$) were activities against *M. smegmatis* and *S. aureus* WT, but the MIC values were greater than 50 μg/ml. The observed EC_{50} values of caffemides **1b** and **1e** were (6.57 μM) and (11.90 μM) respectively, which were less than caffeic acid and vitamin E. Compound **1e** which had tertiary group at the meta position (ring B) with respect to amide linkage almost similar structure with compound **1b**, exhibited antibacterial activity against *M. smegmatis* and *S. aureus* WT bacteria but the MIC value was greater than 50 μg/ml and inactive against on *P. aeruginosa* and *S. aureus* MRSA whereas caffemide **1b** was significantly active towards all tested bacteria. This observation revealed that one hydroxy and one methyl group at ring B ($R^1 = OH$, $R^4 = CH_3$) played an important role for the activity of caffemide **1b**. The opposite pattern was observed for compound **1i** ($R^1 = S\text{-}Ph$) structurally identical with compound **1g** ($R^1 = NH\text{-}Ph$) had higher MIC value against *P. aeruginosa* and *S. aureus* MRSA and inactive on *M. smegmatis* and *S. aureus* WT, whereas caffemide **1g** showed significant activity on *M. smegmatis*, *S. aureus* WT, *P. aeruginosa* and *S. aureus* MRSA with MIC values less than 50 μg/ml.

In summary, a series of novel caffemide were synthesized and their antioxidant and antibacterial properties were measured. The experimental observation reveals that caffemides having more antioxidant property may not be active against specified bacteria but the presence of functional groups of caffemides at ring B plays a crucial role for their antioxidant and antibacterial properties. Compounds **1b** and **1g** had antioxidant property along with antibacterial activity which could be helpful for the treatment of oxidative stress and secondary infection for human. Finally, this experimental study might be helpful for the discovery of new antibiotics in future after detailed SAR analysis.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.10.089.

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