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Evaluation of Bactericidal Action of Methylglyoxal and its Further Potentiation in the Presence of Honey

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ABSTRACT

Despite widespread availability of antibiotics, it is currently advised that clinical administration of antibiotics against pathogenic bacteria should be prohibited due to the emergence of multi-drug resistant bacterial strains. Therefore newer and more effective antimicrobials are in demand to treat such cases. The aldehyde form of pyruvic acid called methylglyoxal has been reported to induce lanthanum-sensitive Ca^{2+} transients for growth inhibition in *Escheriachia coli*. Based on this observation several Gram positive and Gram negative bacteria were tested to determine antibacterial potentiality of methylglyoxal alone and in combination with honey, since methylglyoxal is known to be present in the honey of manuka flowers. Methylglyoxal alone was found to be distinctly active against *Staphylococcus aureus* and also against *E. coli*, salmonellae and vibrios, while *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter boumanii* were less sensitive to this agent. Supplementation of honey augmented antimicrobial property of methylglyoxal to a great extent. Methylglyoxal was found to be highly bactericidal in nature since there was a sharp fall in the number of viable bacteria after addition of methylglyoxal at the logarithmic growth phase and all the cells were killed within one hour. Rod-shaped cells of *E.coli* became round and adhered to each other after treatment with methylglyoxal in one hour.

Keywords: antibacterial, bactericidal, broad-spectrum, methylglyoxal

INTRODUCTION

Methylglyoxal is the aldehyde form of pyruvic acid. Being a dicarbonyl compound containing two carbonyl groups, methylglyoxal is both an aldehyde and a ketone (Cooper 1975). Methylglyoxal is a product from New Zealand manuka honey. As the bees invade the nectar of manuka flowers, a small amount of methylglyoxal and a larger quantity of dihydroxyacetone are formed. On storage at 37°C, however, the amount of methylgyloxal increases coupled with a substantial decrease in the amount of dihydroxyacetone. Dihydroxyacetone when added to clover honey gets converted to methylglyoxal on incubation (Adams *et al*. 2009).

Methylglyoxal had been recognized as a potent anticancer agent since early 1960s (Egyud and Szent-Gtorgyi 1968). Szent-Gyorgyi *et al.* (1967) reported that methylglyoxal is a natural growth regulator and could act as an anticancer agent. They were able to provide strong experimental evidences in favour of this hypothesis. Apple and Greenberg (1967, 1968) successfully determined the remarkable curative effect of methylglyoxal in mice experimentally infected with a variety of cancers. Subsequently, tumoricidal action of methylglyoxal was found to be due to inhibition of glycolysis and mitochondrial respiration of malignant cells (Ray *et al*. 1991; Biswas *et al*. 1997). However, recent studies have revealed that the anticancer activity of methylglyoxal can be further augmented when combined with ascorbic acid and creatinine (Ghosh *et al*. 2006).

In the studies for evaluation of antibacterial effect of methylglyoxal it has been noticed that this compound and its analogues were competent inhibitors of polyamine biosynthesis pathway (Midorikawa *et al*. 1991). Campbell *et al.* (2004) noted that methylglyoxal and other carbohydrate metabolites were able to induce lanthanum sensitive Ca^{2+} transients for inhibition of growth in *E. coli*. On the basis of such observations the present study was designed to determine the antimicrobial potentiality of methylglyoxal alone and in combination with honey against a large number of different bacteria.

MATERIALS AND METHODS

Bacteria

A total of 40 organisms belonging to both Gram-positive and -negative types were taken for the study (**Table 1**).

Agents

A 40% aqueous solution of methylglyoxal was obtained from M. P. Biochemicals, USA. Honey was purchased from Dabur India Ltd in a sealed vial.

Media

Liquid media were nutrient broth (NB, Oxoid), peptone water (PW) containing 1.0% peptone (Oxoid) plus 0.5% NaCl (Analar) and Mueller Hinton broth (MHB, Oxoid). Solid media were nutrient agar (NA, Oxoid) and Mueller Hinton Agar (MHA, Oxoid).

Inoculum

Each organism was grown overnight in NA/MHA at 37°C, harvested during stationary phase and suspended in 5 ml of sterile distilled water. Turbidity of each suspension was matched against 0.5 McFarland standard (Dasgupta *et al*. 2008) in a spectrophotometer at 625 nm corresponding to 2.4×10^8 colony forming units (CFUs/ml).

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Research Note

Table 1 Antibacterial activity of methylglyoxal (Mg) and methylglyoxal plus honey (Mgh) in different bacteria.

Bacteria	Minimum inhibitory concentration (mM/ml)		
	No of test bacteria	Mg	Mgh
Staphylococcus aureus NCTC 6571, NCTC 8530, NCTC 8531, ATCC 25923, ATCC 29737, ML 152, UT007	7	0.5	0.2
S.aureus NCTC 8532, ML 277, ML422, WS 75	4	1.5	0.5
Escherichia coli K12 Row	1	2.0	1.5
E. coli 871, ATCC 25922, K12C600	3	1.5	0.5
Salmonella typhi 59	1	1.0	0.5
S. typhimurium NCTC 74	1	1.0	0.5
S. choleraesuis NCTC 36	1	1.0	0.5
S. abony NCTC 6017	1	2.0	1.5
Shigella flexneri 3b NK 331	1	1.5	0.5
Sh. flexneri 6 895, 20570	2	1.5	1.0
Sh. flexneri 2D DN13	1	2.0	1
Sh. sonnei 2	1	2.0	1
Sh. sonnei DN 9	1	1.5	1
Sh. sonnei NK840	1	2	1.5
Acinetobacter baumannii 470, 517	2	>2	>2
Klebsiella pneumoniae 1	1	2	1.5
K.. pneumoniae J/1/4, R114	2	>2	2
Vibrio cholerae 569B, 590, 820	3	1	0.5
V. cholerae VRC 426/76	1	2	1.5
Pseudomonas aeruginosa APC 1, BVC 3, BVC4, AMRI 100, 732	5	>2	>3

Determination of minimum inhibitory concentration (MIC) of methylglyoxal and methylglyoxal plus honey

This was carried out according to the guidelines of Clinical Laboratory Standards Institute (CLSI 2005) by spot inoculating 10^5 CFU with the help of a 2-mm loop full of 1/10 dilution of 18 hr broth culture on NA and MHA plates containing 0 (control), 0.1, 0.2, 0.5, 1 and 2 mM/ml of methylglyoxal, the dilutions being made in sterile distilled water. Subsequently, all the test bacteria were inoculated in the similar manner on NA and MHA containing the same amounts of methylglyoxal, where the dilutions were made in honey instead of distilled water.

Mechanism of action of methylglyoxal

Two strains, *S. aureus* NCTC 8530 and *E. coli* ATCC 25922 were taken for the study. Each bacterium was grown overnight in 4 ml of NB for 18 h; 2 ml from this culture was given as an inoculum to 4 ml of fresh NB and incubated at 37°C. With respect to each bacterium, as the culture attained logarithmic growth phase after 2 h, CFU/ml count was determined and methylglyoxal was added at twice the amount of its respective MIC value. Subsequently CFU/ml counts were taken after 15 min, 30 min, 45 min, 1 hr, 6 hr and 18 hr. From every time point three CFU counts were taken in three separate NA plates. The data were analysed by determining standard deviation (SD); and the results are presented graphically.

Bacterial cells from the culture were observed at every time point from the beginning (0 hr, before addition of methylglyoxal) in Leica DM 3000 microscope (Leica Microsystems, Switzerland) at 100X magnification.

RESULTS AND DISCUSSION

Antibacterial action of methylglyoxal and methylglyoxal supplemented with honey

Table 1 shows that methylglyoxal exhibited distinct antibacterial function on strains of *S. aureus*. Among 4 strains of *V. cholerae*, 3 were inhibited at 1 mM/ml of the agent. Strains of *E. coli* and salmonellae were moderately sensitive to methylglyoxal, while shigellae were less sensitive. Both the strains of *A. boumanii* and all 5 strains of *P. aeruginosa* were rather resistant to this natural compound. It is clearly noted (**Table 1**) that incorporation of honey in the media could enhance sensitivity of almost all the organisms to a great extent.

Bactericidal action of methylglyoxal

The MIC of methylglyoxal was 1.5 mM/ml in *E. coli* 25922; at the logarithmic growth phase when the CFU/ml count was 6×10^7, 3 mM/ml of methylglyoxal was added and incubated. After 15 min there was a sharp fall in the number of viable cells which continued in the same manner. Although some viable cells could be detected after 45 min, the CFU count became 0 at the end of 1 hr. The results were calculated by SD and are presented in **Fig. 1**. An identical observation was made in *S. aureus* 8530 as well. Typical, rod shaped multiplying *E. coli* cells were seen at 0 hr before addition of methylglyoxal (**Fig. 2**). Many cells were found to have changed their shape and became round at the end of 30 min (**Fig. 3**). Finally, after 1 hr almost all the cells became totally round and adhered to each other forming long chains and clusters of bacteria (**Fig. 4**).

The dicarbonyl compound methylglyoxal has been investigated for many years for its potentiality as an anticancer agent. Although extensive studies have been carried out in tissue culture systems and animal models which have definitely proved its non-toxic nature *in vivo* (Ghosh *et al*. 2006), methylglyoxal is yet to come to market as a commercial product to treat cancer patients.

Methylglyoxal has shown to possess antibacterial activity against a wide range of Gram-positive and Gram-negative organisms with MIC ranging from 0.5 to 2.0 mM/ml in most cases. However, this agent was much more active against 7 strains of *S. aureus* where the MIC was 0.5 mM/ml only. As methylglyoxal was diluted in honey instead of

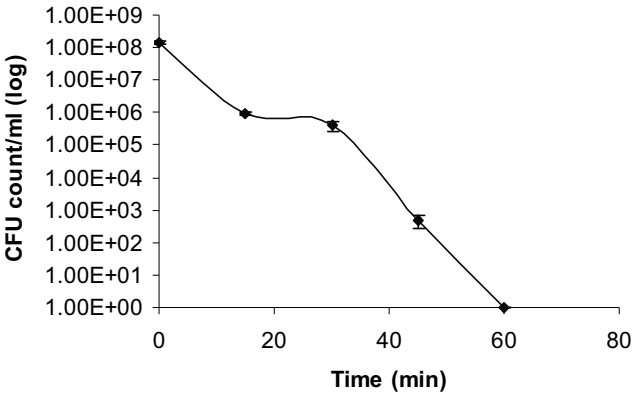

Fig. 1 Reduction in number of bacterial cells (CFU/ml).

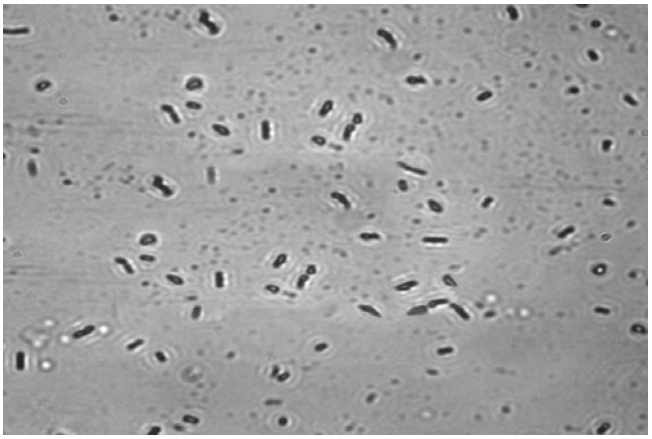

Fig. 2 Shows typical rod-shaped multiplying cells of *E. coli* 25922 at the logarithmic growth phase.

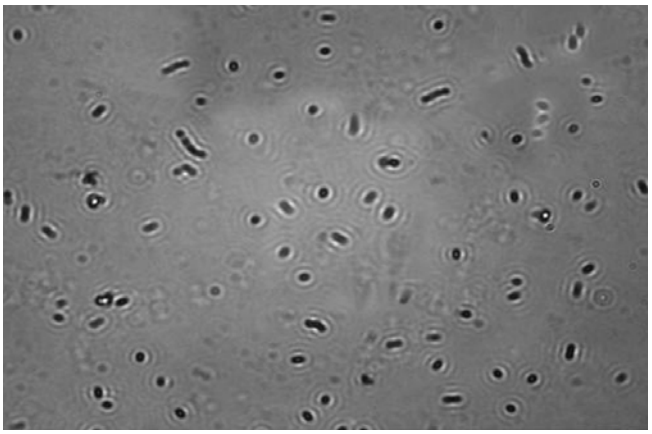

Fig. 3 Rod-shaped cells of *E. coli* are intermixed with round, coccus-like cells, 30 mins after treatment of methylglyoxal.

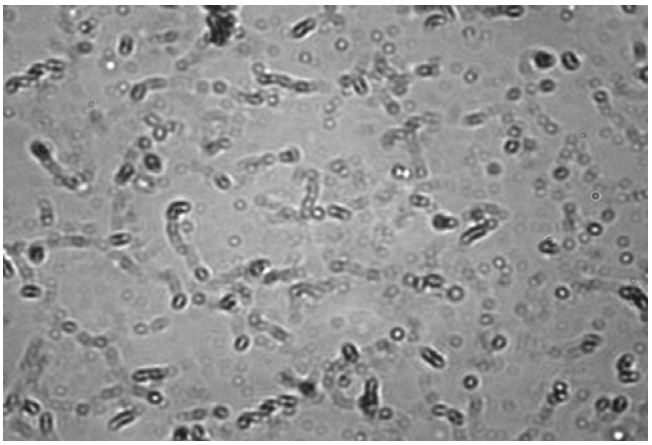

Fig. 4 Round, coccus-like cells seen in pairs, and chains 1 hr after treatment of *E. coli* culture with methylglyoxal.

distilled water, its activity increased 2- to 3-fold against almost all the test strains (**Table 1**). While sensitive organisms were *S. aureus*, *E. coli*, salmonellae and vibrios, strains of *K. pneumoniae*, *A. boumanii* and *P. aeruginosa* showed resistance, even when methylglyoxal was supplemented with honey. Methylglyoxal is intensively bactericidal against both Gram positive and Gram negative types. The number of live cells started decreasing within 15 minutes after addition of this agent to a culture in its logarithmic growth phase and all the cells were found to perish within 1 hr. The bactericidal action appears to be so intense that methylglyoxal probably forces the cellular materials to come out of the cells making them rather look like cocci. The cells then became attached to each other forming chains or clusters possibly due to the stickiness of the cellular materials inside the cells.

It has been observed that methylglyoxal has a distinct inhibitory effect on the growth rate of *E. coli* and the extent of inhibition was found to be dependent not only on the concentration of methylglyoxal, but also on the cell density of the culture (Szent *et al*. 1967). Hadrian *et al.* (1980) further reported that a substantial increase in the amount of both this agent and density of cells in the culture could result in complete inhibition of growth. They also noted that addition of methylglyoxal prior to or at the time of initiation of DNA synthesis could completely stop the DNA chain elongation process. According to them complete inhibition of protein synthesis occurred immediately after addition of methylglyoxal, which was possibly linked to the blockage of DNA replication. These observations of earlier workers entirely corroborate with our data on powerful bactericidal action of this natural compound. Thus this study opens up a new avenue in the search for potent antibacterial drugs. Structurally methylglyoxal is a very simple compound and can therefore be easily synthesized in a pharmaceutical industry.

CONCLUSIONS

In this era of search for new antimicrobial agents by both medical research institutions and the giant commercial industries an uncomplicated easy to synthesize antimicrobial agent, such as methylglyoxal, can be the immediate solution for the emerging problem of multi-drug resistances among almost all the human pathogens. Further studies are in progress. Extensive and in-depth analysis on methylglyoxal activity may to lead to its establishment as a powerful drug for multidrug resistant pathogens in the long run. Therefore, manufacture and application of this proven non-toxic agent is likely to open up a completely new regime of treatment. Since honey has been found to enhance the action of methylglyoxal, the newly synthesized compound in the industry may be made available for human consumption along with honey.

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