

Comparison of the disc diffusion assay with spectrophotometer technique for antibacterial activity of diclofenac sodium, indomethacin and mefenamic acid

Ali Abdul Hussein S AL-Janabi

Department of Clinical Laboratory, Collage of Pharmacy, University of Karbala, Karbala, Iraq

Non-steroidal anti-inflammatory drugs (NSAIDs) contain a wide range of effective drugs that use in treatment of inflammatory diseases. Chemical agents of non-steroidal anti-inflammatory drugs including diclofenac sodium, indomethacin and mefenamic acid, were screened for antibacterial activity by using two methods: Disc diffusion assay and spectrophotometer technique. From the results of disc diffusion method, *Staphylococcus aureus* exhibited susceptibility to diclofenac sodium and mefenamic acid. While, spectrophotometer method showed that *Paracoccus yeei* was susceptible to all tested compounds. Diclofenac sodium had loweress MIC (2.5 mg/ml) to inhibit all of seven isolated strains. The application of spectrophotometer technique in this study considers more suitable method than those of disc diffusion test to reveal antibacterial activities of tested agents, especially with indomethacin.

Key words: Diclofenac sodium, indomethacin, mefenamic acid, spectrophotometer

INTRODUCTION

Among non-steroidal anti-inflammatory drugs (NSAID) group, diclofenac sodium, indomethacin and mefenamic acid are commonly used. Thus, production volume of these drugs estimated to be in the hundreds of tons per year.^[1] The pharmacologic applications of these drugs are for analgesic, antipyretic, antiarthritis and anti-rheumatic action.^[2] Antimicrobial effects of diclofenac had been proved by many studies. Diclofenac showed noteworthy inhibitory action [MIC_{90}] = 50 $\mu\text{g/ml}$) on *Listeria monocytogenes*, with demonstrated cidal activity on this bacteria at 100 $\mu\text{g/ml}$.^[3] A total of 80 isolates of *E. coli* from UTIs patients were susceptible to diclofenac at MIC value ranging from 5-50 $\mu\text{g/ml}$.^[4] Moreover, most of 45 strains of *Mycobacterium tuberculosis* were inhibited by diclofenac sodium at concentrations of 10-25 $\mu\text{g/ml}$ when tested *in vitro*.^[5] Meanwhile, the susceptibility of the same bacteria (*M. tuberculosis*) to mefenamic acid had been estimated at 33 mg/L in the pH 6.8 and at 11mg/L in pH 5.5 compared with the effect of

mefenamic acid on *M. smegmatis* which was found at 1000 and 333mg/L in pH 6.8 and pH 5.5, respectively.^[6]

The activity of drug on bacteria may differ based on bacterial species or strain. Therefore, the injection of indomethacin into rat gastrointestinal increased the persistence of *Enterococci faecalis* and decreased *E. coli* growth in the same time.^[7]

The antimicrobial ability of diclofenac sodium, indomethacin and mefenamic acid to eliminate pathogenic organisms is not limited with direct inhibitory action on those organisms, but also includes indirect effects by using the main function of such compounds as anti-inflammatory to facility the destruction of affected organisms. Therefore, diclofenac sodium and indomethacin have removal capacity of gram negative bacteria from kidney through effects on the function of mucosal inflammatory response represented by secretion of interleukin-6 and polymorphonuclear leukocytes (PMNL).^[8] In meningitis patients, Diclofenac sodium and indomethacin reduce the inflammation resulted from infection with bacterial meningitis.^[9]

For illustrating any possible pharmaceutical activities of diclofenac sodium, indomethacin and mefenamic acid on microorganisms, the antibacterial action of these compounds was investigated in this study against many isolated strains of pathogenic bacteria.

Address for correspondence:

Dr. Ali Abdul Hussein S AL-Janabi, College of Pharmacy,
University of Karbala, Karbala, Iraq
E-mail: aljanabi_bio@yahoo.com

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MATERIALS AND METHODS

Organisms

A total of seven recent clinical bacterial strains were isolated from the skin and stool of patients (28-35 years) at AL-Hussein general hospital of Karbala city. Samples were cultured on Mueller-Hinton agar and blood agar (HiMedia, Mumbai, India) and incubated at 35 C for 24 hrs. Diagnosis of isolated strains was performed using API 20 system (Biomérieux, Netherlands-France) with the criteria of morphology and type of gram stain

The diagnosed strains were; *Staphylococci aureus*, *E. coli*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Salmonella typhi* and *Paracoccus yeei*.

Chemical agents

Diclofenac sodium, indomethacin and mefenamic acid were supplied by Brown and Burk pharmaceutical Limited Bangalore-India. Ampicillin sodium and cefotaxime sodium were supplied by KonTam pharmaceuticals co. Zhongshan-China.

Inoculate and preparations agentes

Isolated strains were inoculated in Mueller-Hinton broth. Inoculum suspension was standardized (0.5 MacFarland reading to 1×10^8 cfu/ml) and diluted 1:10.

For obtaining stock solutions, indomethacin and mefenamic acid were dissolved in methanol. Diclofenac sodium, ampicillin sodium and cefotaxime sodium were dissolved in sterilized distill water.^[2] Drug concentrations were serial two-fold dilutions ranging from 5 to 0.078 mg/ml.

Antimicrobial assay

Disc diffusion assay

Disc diffusion test was performed according to NCCLS disc diffusion method.^[10] Briefly, filter paper discs (6 mm) were prepared from Whatman No. 1 and sterilized by dry oven at 140C for 60 min. Prepared discs with different concentrations of tested agents were placed on the surface of inoculated Mueller-Hinton agar. Plates were incubated at 35 C for 24 hrs and zone of inhibition was measured (mm).

Spectrophotometer technique

Various concentrations of tested agents were prepared in tube with Mueller-Hinton broth. Tubes with tested agents were inoculated with standard culture of each strain (50 μ l to each milliliter of broth). All tubes were incubated at 35 C for 24 hrs. Optical density of grown bacteria was measured by spectrophotometer (Optima-SP-300; Kartzma Co. Tokyo' Japan), at wavelength of 450 nm.^[11]

Three main controls were included in previous methods: Ampicillin sodium and cefotaxime sodium at concentrations

of 20, 39, 78 μ g/ml. Chemical-free medium (control) was also included. Each experiment was repeated three times with triplicates of each concentration for statistical analysis.

Determination of minimal inhibitory concentrations

All organisms were tested by the broth microdilution method recommended by the NCCLS.^[12] Briefly, drug concentrations tested were serial two-fold dilutions in Mueller-Hinton broth. Each well of microdilution tray (96 wells) was received 100 μ l of mixed broth media. Inoculum (50 μ l) was added to each well. Trays were incubated at 35 C for 24 hrs and examined for visible growth. Ampicillin sodium, cefotaxime sodium and free media were also included as control.

Statistical analysis

Result data were statistically analyzed by using two-way variance of analysis (ANOVA) with less significant difference (L.S.D.) at $P < 0.05$.

RESULTS

For detection of a potential antibacterial effect of diclofenac sodium, indomethacin and mefenamic acid, two methods were applied: Disc diffusion assay and spectrophotometer technique.

According to disc diffusion method, diclofenac sodium was able to inhibit four strains of bacteria at lower concentrations. Whereas, three strains including *E. coli*, *Ent. aerogenes* and *S. aureus* were needed more concentrations of mefenamic acid to decrease their growth ratio. *S. aureus* could be considered the most susceptible bacteria to diclofenac and mefenamic acid than other strains. All seven isolates were exhibited resistance to indomethacin up to 5 mg/ml [Table 1].

Spectrophotometer assay gave much more valuable value about the inhibitory action of tested chemical agents. Diclofenac sodium also considered the powerful compound on tested bacteria. Comparing with control, the growth of all isolates was significantly reduced by 2.5 mg/ml (MIC) of diclofenac sodium [Table 2] Meanwhile, *P. yeei* tend to be the most susceptible strain to lower level of diclofenac followed by *B. subtilis* and *S. aureus* [Figure 1]. Unlike the results of disc diffusion, indomethacin showed antibacterial activity with significant effect against most strains, especially on *P. yeei*, *Sal. typhi* and *E. coli* [Figure 2] After statistical analysis, the same three strains (as with indomethacin) also exhibited susceptibility to mefenamic acid in comparing with the growth of bacteria on free compound medium (control) [Figure 3].

From other hand, mefenamic acid in broth media enhanced the growth of some tested strains, especially *S. aureus*, *B. subtilis* and *Ent. aerogenes* [Figure 3].

Table 1: Effect of diclofenac sodium, indomethacin and mefenamic acid on isolated strains determined by disc diffusion method

Strains	Diclofenac sodium				Indomethacin		Mefenamic acid		
	Zone of inhibition (mm)				5	2.5-0.315	5	2.5	1.25-0.315
	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.615-0.315 mg/ml	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
<i>E. coli</i>	R	R	R	R	R	R	9	R	R
<i>Sal. typhi</i>	R	R	R	R	R	R	R	R	R
<i>Ent. cloacae</i>	R	R	R	R	R	R	R	R	R
<i>Ent. aerogenes</i>	10	9	R	R	R	R	9	R	R
<i>S. aureus</i>	18	15	7	R	R	R	8	7	R
<i>B. subtilis</i>	12	11	R	R	R	R	R	R	R
<i>P. yeei</i>	9	8	R	R	R	R	R	R	R

R: Resistant

Table 2: MICs of diclofenac sodium, indomethacin and mefenamic acid for isolated strains

Chemical agents	Conc. (mg/ml)	Strains						
		<i>E. coli</i>	<i>Sal. typhi</i>	<i>Ent. cloacae</i>	<i>Ent. aerogenes</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>P. yeei</i>
Diclofenac sodium	5	-	-	-	-	-	-	-
	2.5	-	-	-	-	-	-	-
	1.25	+	+	+	+	-	-	-
	0.615	+	+	+	+	-	-	-
	0.315	+	+	+	+	+	-	-
	0.56	+	+	+	+	+	+	-
	0.078	+	+	+	+	+	+	+
Indomethacin	5	+	+	+	+	+	+	+
	2.5	+	+	+	+	+	+	+
	1.25	+	+	+	+	+	+	+
	0.615	+	+	+	+	+	+	+
	0.315	+	+	+	+	+	+	+
	0.56	+	+	+	+	+	+	+
Mefenamic acid	5	+	+	+	+	+	+	+
	2.5	+	+	+	+	+	+	+
	1.25	+	+	+	+	+	+	+
	0.615	+	+	+	+	+	+	+
	0.315	+	+	+	+	+	+	+
	0.56	+	+	+	+	+	+	+

- = No growth; + = Growth

DISCUSSION

Large amounts of diclofenac sodium, indomethacin and mefenamic acid are consuming every day all over the world for treatment of many inflammatory diseases. Obtaining data about antibacterial action of such drugs is still unclear due to variability of influencing factors. Type of employed assay that may use to illustrate drug activity is one of these factors.

In comparing with other methods, disc diffusion test that recommended by NCCLS demonstrated to have the ability for evaluating the activity of many antimicrobial agents, including diclofenac sodium^[5] and mefenamic acid.^[13]

However, the results obtained from application of this method, in some studies, has no significant differences from other antibacterial assays.^[14]

Spectrophotometric method is another technique that can perform for determining the inhibitory action of compounds on microorganisms. It has much quicker and easier to use and to replicate. Domínguez *et al.*^[11] found no statistically significant differences between the results obtained with the spectrophotometric method and viable count of bacteria in agar.

In recent study, the demonstration of antimicrobial ability of three chemical agents was illustrated by manipulation of two methods. Spectrophotometer assay can recommend to be the most suitable and sensitive technique that may apply to investigate the antimicrobial activity of diclofenac sodium, indomethacin and mefenamic acid.

The susceptibility of bacteria to inhibitory action of tested compounds is variable based on bacterial strain. *P. yeei* exhibited much more susceptible to three agents than that

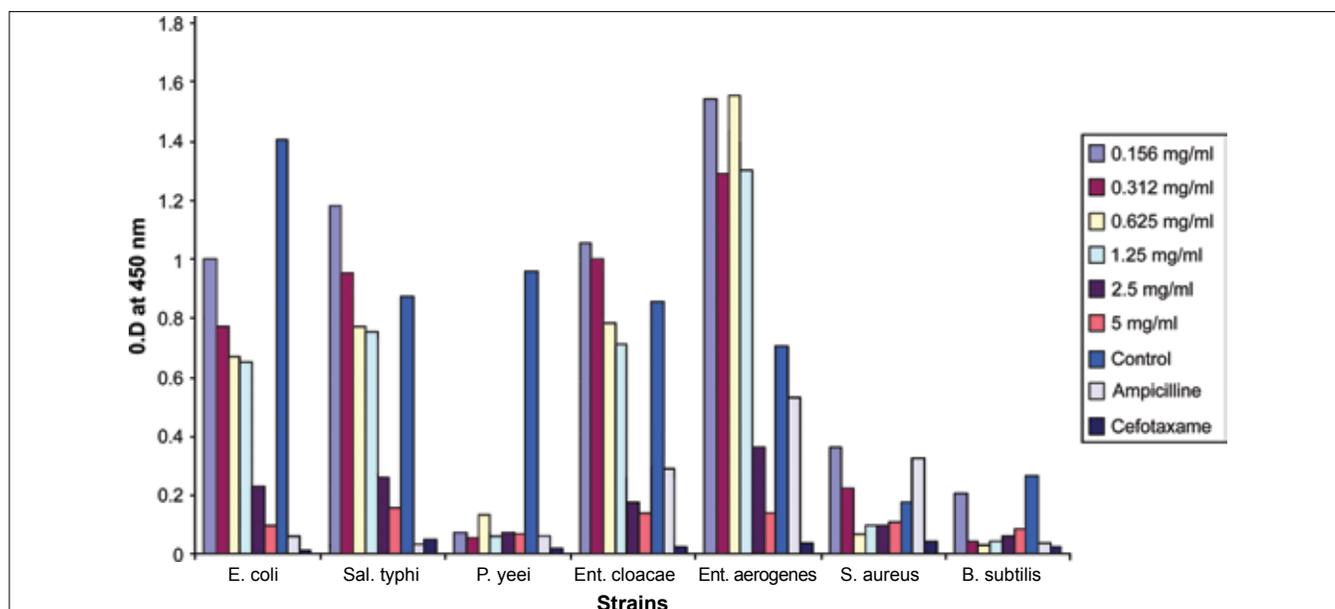


Figure 1: Effect of different concentrations of diclofenac sodium on the growth of isolated strains

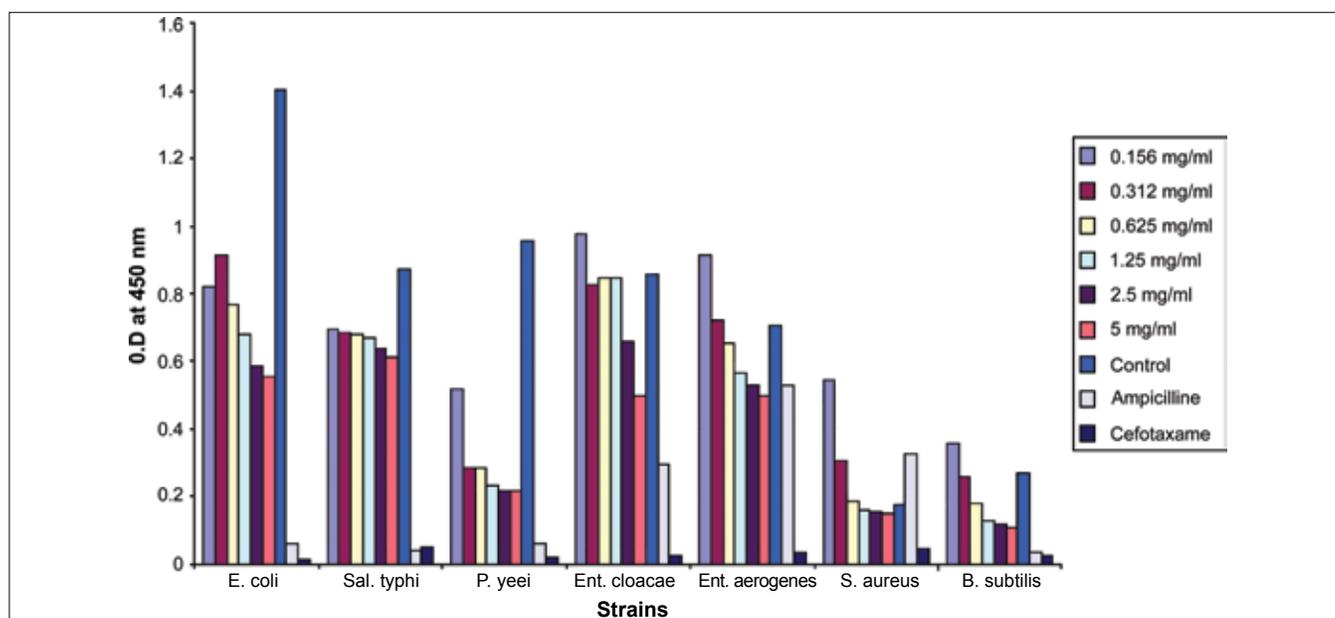


Figure 2: Effect of different concentrations of indomethacin on the growth of isolated strains

of other strains. Kruszewska *et al.*^[13] found no activity of mefenamic acid on growth of *E. coli* while the isolated *E. coli* in present study showed susceptibility to mefenamic acid.

Although diclofenac sodium recorded to have antimicrobial effect on many species of bacteria,^[3-5] other studies improved the absence of this activity, such as with *Cytophaga*, *Flavobacterium* and *Y-Proteobacteria* group.^[1]

The results of two application methods about antimicrobial action of indomethacin were ranged between effective and noneffective. Disc diffusion test revealed no activity of indomethacin, while spectrophotometer assay gave reversible

results by showing significant effect of indomethacin on some isolated strains. The possible explanation for these variable results may related to poor solubility of indomethacin in water^[2] which may decrease the diffusion ratio of indomethacin from prepared filter disc to media. Mefenamic acid solubility in water is also limited but much higher than with indomethacin. The solubility of these agents may increase when complexes with other compounds, such as association of mefenamic acid with β -cyclodextrin^[15] or conversion of indomethacin to indomethacin sodium trihydrate.^[2]

The mechanism of action of diclofenac sodium, indomethacin and mefenamic acid in human body is indicated by decrease

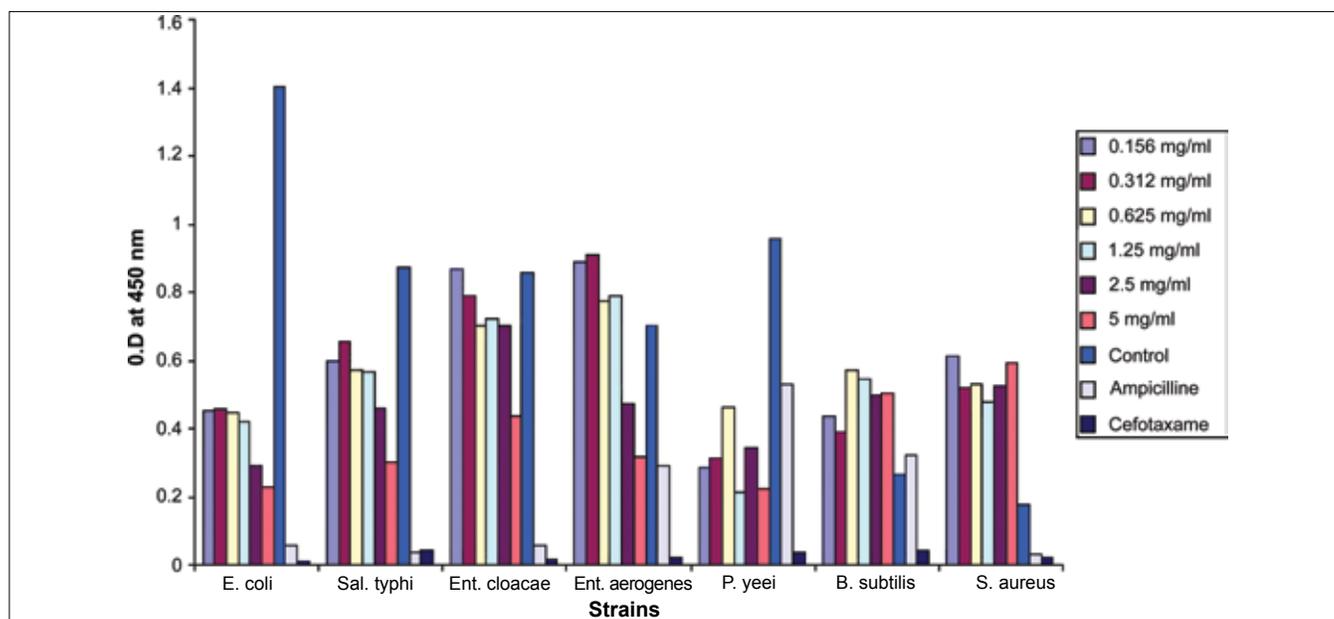


Figure 3: Effect of different concentrations of mefenamic acid on the growth of isolated strains

of the prostaglandin synthesis from arachidonic acid by inhibition of the cyclo-oxygenase activity.^[15,16]

In cells of *Listeria monocytogenes*, the site of action of diclofenac had been determined by inhibition of DNA synthesis and damaged the bacterial membrane.^[3]

According to our results, the inhibitory action of mefenamic acid on some strains had been converted to enhancement action through increasing the growth of some strains. This elevation in growth ratio of bacteria may result from the ability of some bacteria to destruct mefenamic acid and use degrades for synthesis of their nucleic acid due to the similarity in structure between mefenamic acid and purine group.^[2]

In conclusion, spectrophotometer technique is efficient method to determine antibacterial action of diclofenac sodium, indomethacin and mefenamic acid. Diclofenac sodium seems to be effective to inhibit the growth of bacteria in lower concentrations. Whereas, mefenamic acid may play an enhancement factor for growing some bacteria in addition to its inhibitory action. Conversion the effect of some NSAID compounds on immune system to antimicrobial agents needs many scientific evidences to form clear view on this activity against various microorganisms.

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