Synthesis, antimicrobial and antifungal possessions of tramadol esters: *In vitro* studies

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Abstract: Tramadol esters were prepared by refluxing equimolar concentration of tramadol with leucine and asparagine separately with methanol, sulphuric acid and phthalic anhydride for 10 hours and temperature was maintained at 75°C. After refluxing, the colour of sample solutions were changed from colorless to yellow, blank solution was prepared in the same way as the sample solution except the Tramadol. Both the products and blank were neutralized with sodium carbonate and excess of sodium bicarbonate was precipitated as sodium sulphate, which was washed with acetone. The structures of both the products were confirmed with spectral data (FT-IR, ^{1}HNMR and ^{13}CNMR). Antimicrobial and anti-fungal property of derivative of analgesic tramadol drug was tested with one fungus and three sensitive bacteria belonging to both gram positive and gram-negative types. Esterified product of tramadol with leucine and asparagine showed moderate activity against *Escherichia coli* and *Tricophyton rubrum*. Both the products showed marked activity against *Staphylococcus aureus* and found no activity against *Salmonella spp.*

Keywords: Inhibition zone; bacterial strains; esterification.

INTRODUCTION

Tramadol is a synthetic phenanthrene alkaloid analog of codeine that binds to μ-opioid receptors and inhibits nor epinephrine and serotonin reuptake, also has local anesthetic properties and is beginning to be used to extend the duration of analgesia of peripheric nerves blocks (Hanife *et al.,* 2004; Robaux *et al.,* 2004). Antihistamines, tranquilizers, antihypertensive, antipsychotics and the anti-inflammatory agents belonging to different pharmacological categories showed moderate to extraordinary antimicrobial actions (Kristiansen *et al* 1987; Chattopadhyay *et al.,* 1988; Dastidar *et al* 1995). Such compounds, having antimicrobial properties in adding to their predestinated pharmacological trial, have been dedicated as non-antibiotics (Dastidar *et al.,* 1986; Manna *et al.,* 2001; Annaduri *et al.,* 2008). The antibacterial activity of tramadol against *E. coli* and *S. epidermidis*, *S. aureus* and *P. aeruginosa* have been done respectively which was time-dependent. The study indicated that antibacterial properties of tramadol may be useful for reducing the risk of bacterial infection after local or regional anesthesia (Martine *et al.,* 2007). The infection complications can be caused by the gram-negative bacteria like *E. coli* and *Salmonella spp*. and gram positive bacteria like *S. aureus* (Du Pen *et al.,* 1990; Feldman *et al.,* 1994). Aim of this study was to synthesize and investigate the *in vitro* antibacterial and anti-fungal activity of tramadol alone and esters of tramadol with leucine and asparagine against *S. aureus, Salmonella spp*., *E. coli* and *Trichophyton rubrum*.

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MATERIALS AND METHODS

Reagents and chemicals

Nutrient agar (E. Merck), Sabouraud culture media, (E. Merck), Asparagine (The British Drug House), Leucine (The British Drug House), Dimethyl sulphoxide (E. Merck), Sulphuric acid (E. Merck) Methanol (E. Merck), Tramadol (Java pharmaceutical), Sterile water (Surge Pharma), Phthalic anhydride (E. Merck).

Bacterial strains and fungus

Escherichia coli, Salmonella spp., Staphylococcus aureus and Trichophyton rubrum were collected from Mycology Department, University of Punjab, Lahore, Pakistan were maintained in tryptic soy agar (TSA) and Sabouraud culture media slants at 5°C until use.

Media for bacterial strains and fungus

The media used for *Escherichia coli, Salmonella spp.* and *Staphylococcus aureus* was tryptic soy agar and sterilized by heating in an autoclave at 121°C for 30 min with 15psi pressure after adjusting the pH at 7.1. Sabouraud culture media used for Optimal fungal growth (*Trichophyton rubrum*) due to their relative high carbohydrate concentrations (4%) sterilized by heating in an autoclave at 121°C for 30 min with 15psi pressure after adjusting pH: 5.6±0.2 at 25°C.

Synthesis of tramadol ester

10ml of 0.1M solution of leucine, 10ml of 0.1M solution of Tramadol, 10ml of methanol, 1ml of H_2SO_4 and 0.5g of phthalic anhydride were added to 2-necked flask of 250ml. The contents of both flasks were refluxed for 10 hours and temperature was maintained at 75°C. Similarly,

10ml of 0.1M solution of Asparagine salt, 10ml of 0.1M solution of Tramadol10ml of methanol, 1ml of H_2SO_4 and 0.5g of phthalic anhydride were added to 2-necked flask of 250ml. The content of both flasks were refluxed for 10 hours and temperature is maintained at 75°C. After refluxing, the colour of sample solutions were changed from colorless to yellow, blank solution was prepared in the same way as the sample solution except the Tramadol. Perkin Elmer FTIR A-100, NMR spectrometer, Bruker were used to record the IR and ^{1}HNMR, ^{13}CNMR spectra respectively. PG-T80$^+$ UV-Vis spectrophotometer, Flash HT Plus elemental analyzer, Thermo Scientific were used for λ_{max}, and concentration of carbon (C), hydrogen (H) and nitrogen (N) of synthesized compounds respectively while the melting point was measured by Gallenkamp apparatus for validation and identification of tramadol derivative formed.

Neutralization

The sample products of both solutions were neutralized with Na_2CO_3 then contents of both the beakers were filtered separately. The excess of H_2SO_4 by reacting with Na_2CO_3 was concentrated into Na_2SO_4 which remained as residue on filter paper. The neutralized filtrate was obtained in a beaker and the residue was washed with acetone and washing was added to filtrate. The blank solution was also neutralized as sample solution.

Solutions preparation

A series of two 10-fold dilutions of leucine, asparagine and tramadol were made by dissolving 10µg of each in 0.8mL distilled H_2O + 0.2µg of Conc. H_2SO_4 and distilled water respectively. While two 10-fold dilutions were made by dissolving 10µg of tramadol leucine ester and tramadol asparagine ester separately in 1mL Dimethyl sulphoxide (DMSO). All the dilutions were made sterile in an autoclave at 121°C for 30 min with 15psi pressure.

RESULTS

The main IR-peaks observed for tramadol and its derivatives are presented in table 1.

Table 1: Characteristics peak of FTIR spectra of tramadol and esters

Sample	FTIR peaks (cm$^-$)
Tramadol	2931.01
Tramadol-leucine ester	1738.0
Tramadol-asparagine ester	1744.0

The formation of both the compounds was also confirmed by elemental analysis and measuring the absorption maximum (λ_{max}), the hyper chromic effect (272nm for tramadol while 390 nm for ester formed) justified the esterification process.^{1}HNMR and ^{13}CNMR were performed for structure confirmation of both the compounds by dissolving in the DMSO-d_6 (Table 2 & 3). Three singlet signals of methyl group protons were observed in 2.11-3.56 ppm chemical shift values of Tramadol-asparagine while 0.98-3.56 ppm with two doublets for Tramadol-leucine compound. A sharp signal of NH_2 group was also visible in ^{1}HNMR spectrum for both the compounds. The characteristics carbonyl signal δ 174.11 ppm (C-8, C-25) for Tramadol-asparagine and δ 174.11 ppm (C-8) for Tramadol-leucine was showed by ^{13}CNMR.

Also their antimicrobial and antifungal activities were studied. The antimicrobial and antifungal activity of Leucine, Asparagine, Tramadol, Ester of Tramadol with Leucine and asparagine against *Escherichia coli*, *Salmonella spp.*, *Staphylococcus aureus* and *Tricophyton rubrum* respectively presented in table 4 and zone of inhibition are shown in figure 1, 2, 3 and 4 respectively.

Table 2: Physiochemical data of esters of tramadol

Compounds	M.P. (^{0}C)	Yield (%)	λ_{max} (nm)	Molecular formula (Mol. Wt.)	Elemental analysis (found/cal.) %		
					C	H	N
Tramadol - Asparagine ester	201-203	82.9	390	$C_{20}H_{31}N_3O_4$ (377.48)	63.51/63.65	8.26/8.28	11.15/11.13
Tramadol - Leucine ester	211-213	89.3	387	$C_{22}H_{36}N_2O_3$ (376.53)	70.11/70.18	9.64/9.66	7.45/7.43

Table 3: ^{1}H NMR and ^{13}C NMR of the prepared compounds

Compounds	^{1}H NMR δ (ppm)	^{13}C NMR δ (ppm)
Tramadol - Asparagine ester	2.11 (3H, s, J = 13.29 Hz, CH_3), 2.18 (2H, d, J = 13.47, CH_2), 4.19 (1H, t, J = 10.96, CH), 7.33 (2H, J = 2.29 Hz, NH_2), 2.48 (2H, d, J = 15.48, CH_2), 3.56 (3H, CH_3)	81.82 (C-1), 39.11 (C-2), 27.28 (C-3), 174.11 (C-8), 142.9 (C-10), 111.75 (C-11), 55.75 (C-22)
Tramadol - Leucine ester	2.11 (3H, s, J = 13.29 Hz, CH_3), 2.38 (2H, d, J = 13.47, CH_2), 1.59 (1H, t, J = 9.96, CH), 7.03 (2H, NH_2), 0.98 (3H, d, J = 6.38, CH_3), 3.56 (3H, J = 9.46 CH_3)	81.82 (C-1), 37.11 (C-2), 28.48 (C-3), 177.51 (C-8), 142.1 (C-10), 111.95 (C-11), 55.15 (C-22)

Table 4: Antibacterial and Antifungal activity of Leucine, Asparagine, Tramadol, Ester of Tramadol with Leucine and asparagine by Kirby Bauer method

Compounds	Dilutions	Diameter of zone of inhibition (mm ± SD)			
		E. coli	*Salmonella spp.*	*S. aureus*	*T. rubrum*
Leucine (G)	G (1)	11.2±0.30	19.2±0.68	16.2±1.30	13.2±0.76
	G (2)	16.3±0.70	13.3±0.11	13.3±0.90	-
	G (3)	13.4±0.90	12.4±1.01	11.7±0.60	-
Asparagine (Y)	Y (1)	-	12.3±0.91	-	12.3±1.30
	Y (2)	-	-	-	10.4±1.20
	Y (3)	-	-	-	-
Tramadol (Z)	Z (1)	12.09±.06	-	11.2±0.34	-
	Z (2)	-	-	-	-
	Z (3)	-	-	-	-
Tramadol - Asparagine ester (X)	X (1)	14.1±0.21	-	14.2±0.64	13.5±0.91
	X (2)	13.3±0.43	-	13.3±0.78	10.6±0.76
	X (3)	-	-	11.4±0.98	10.5±0.87
Tramadol - Leucine ester (V)	V (1)	14.03±0.61	15.7±1.53	25.2±0.54	13.4±0.92
	V (2)	13.8±0.66	-	13.3±0.75	12.7±0.61
	V (3)	12.6±0.3	-	9.7±0.65	-

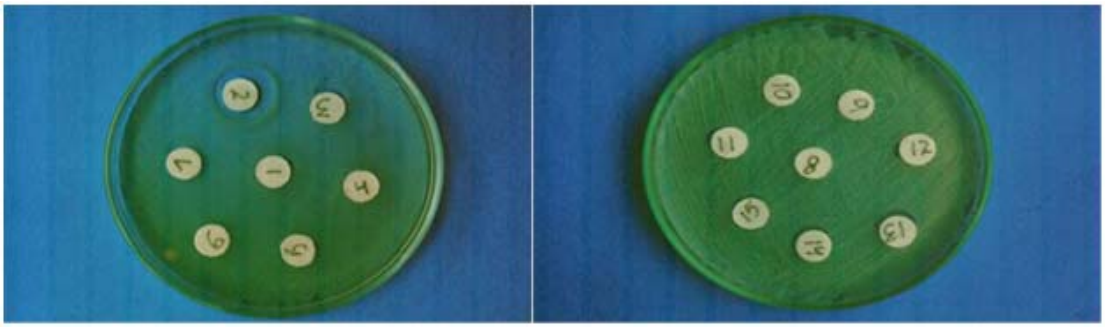

Fig. 1: Zone of inhibitions by activity of compounds against *Escherichia coli*

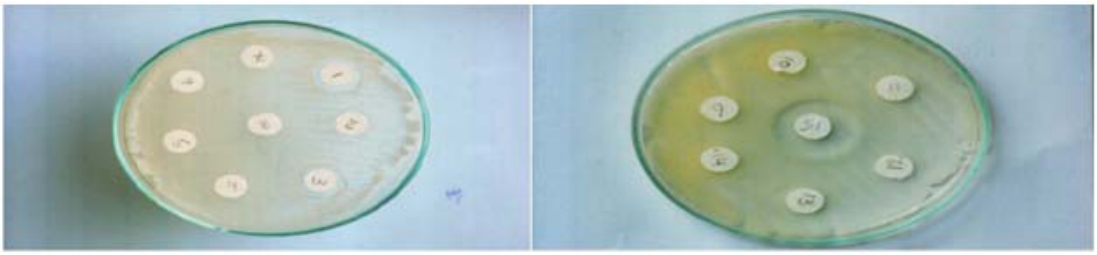

Fig. 2: Zone of inhibitions by activity of compounds against *Salmonella spp.*

Escherichia coli, Staphylococcus aureus and *Tricophyton rubrum* were found to be more sensitive to Ester of Tramadol with asparagine and Leucine with zone of inhibitions 14.1±0.21 & 14.03±0.6 114.2±0.64 & 25.2±0.54 and 13.5±0.91 & 13.4±0.92 respectively but only Tramadol-Leucine ester showed activity against *Salmonella spp.* with zone of inhibition 15.7±1.53.

DISCUSSION

The Kirby Bauer method was used for the antibacterial and antifungal activities.

The effectiveness of antimicrobial and antifungal sensitivity testing is based on the size of the zone of inhibition. The zone of inhibition however, varies with the infusibility of the agent, the size of the inoculums and the type of medium. The zone diameters were measured with a metric ruler in millimeters for result interpretation. In the present study, we demonstrated the antibacterial activity of Tramadol esters with Asparagine and Leucine against gram-negative *Escherichia coli, Salmonella spp.* and gram-positive *Staphylococcus aureus* bacteria and antifungal activity against *Tricophyton rubrum*. Tramadol-Leucine ester demonstrated the highest antibacterial activity against *S. aureus.* It was also noted that greater antibacterial potential possessed by Tramadol-Leucine ester for all bacterial strains and fungus. However, Tramadol-Asparagine showed no antibacterial activity against *Salmonella spp.* at any concentration. The

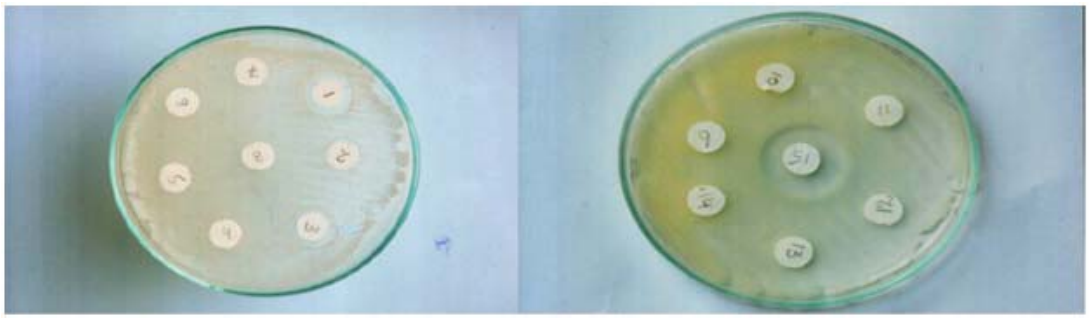

Fig. 3: Zone of inhibitions by activity of compounds against *Staphylococcus aureus*

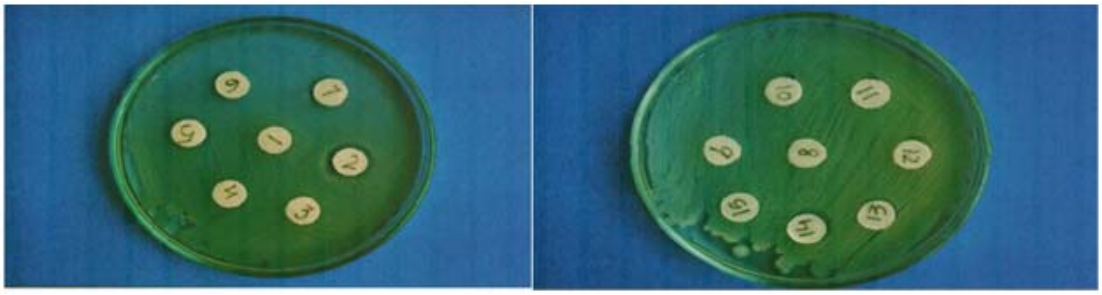

Fig. 4: Zone of inhibitions by activity of compounds against *Tricophyton rubrum*

maximum zone of inhibition was showed by Gram-positive *S. aureus* among all other microorganisms, which found to be the most sensitive bacterial strain to both the esters.

CONCLUSION

In conclusion, the esters of Tramadol were synthesized and their structures were confirmed by spectral analysis. The antibacterial and antifungal activities of esterified products were evaluated against gram negative and gram-positive bacteria and *Tricophyton rubrum*. The present study suggested that esters of Tramadol with Asparagine and Leucine indicated moderate activity against *Escherichia coli* and *Tricophyton rubrum*. Enhanced activity found against *Staphylococcus aureus*. No activity found against *Salmonella spp*. by Tramadol-Asparagine ester.

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