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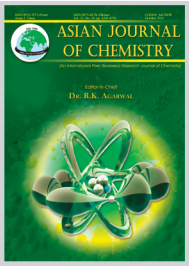

Synthesis, Characterization and Biological Evaluation of Amino Acid Conjugates of Mefenamic Acid

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Mefenamic acid is used for its antipyretic, analgesic, antiinflammatory properties and also inhibits cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2) enzymes reversibly, which decreases production of pro-inflammatory prostaglandin precursors. Mefenamic acid suffers from the general side effects of non-steroidal antiinflammatory drugs, owing to presence of free carboxylic acid group. The study aimed to retard the adverse effects of gastrointestinal origin. Amino acid conjugates of mefenamic acid were synthesized by amidation with methyl esters of amino acids namely, phenylalanine, cysteine, glycine, lysine, arginine, valine, proline, serine, alanine and methionine. Synthesized conjugates were characterized by melting point, TLC, ^{1}H NMR and mass spectroscopy. Synthesized conjugates were also evaluated for their analgesic and antiinflammatory activities. Comparable analgesic and anti-inflammatory activities were obtained as compared to mefenamic acid.

Key Words: Synthesis, Mefenamic acid, Amino acid conjugates, Analgesic, Antiinflammatory activity.

INTRODUCTION

The non-narcotic analgesics have a peripheral site of action and have no affinity for the opioid receptors[1]. Mefenamic acid chemically is 2-(2,3-dimethylphenylamino) benzoic acid derivative and it acts as analgesic, antipyretic and antiinflammatory drug, which inhibit COX as well as antagonizes certain actions of prostaglandin[2]. Mefenamic acid exerts peripheral as well as central analgesic action. Mefenamic acid appears to be rapidly absorbed from the gastrointestinal tract following oral administration to humans[3]. Mefenamic acid is prescribed in the treatment of rheumatoid arthritis, osteoarthritis and other joint diseases[4]. It is used to relieve pain and inflammation in a wide range of musculoskeletal conditions, including rheumatoid arthritis, muscular pain and traumatic pain such as sprains and fractures. It can also be used to relieve other types of pain such as toothache and headaches[1].

The major factor, which limits the use of mefenamic acid, is its gastric effect due to local irritation of gastric mucosa by free -COOH group of the drug[5]. Hence efforts have been made to mask the free -COOH group by methyl ester of amino acids.

The purpose of this study was to mask the free acidic group of mefenamic acid by synthesizing its amino acid conjugates and evaluation of their analgesic and antiinflammatory activities.

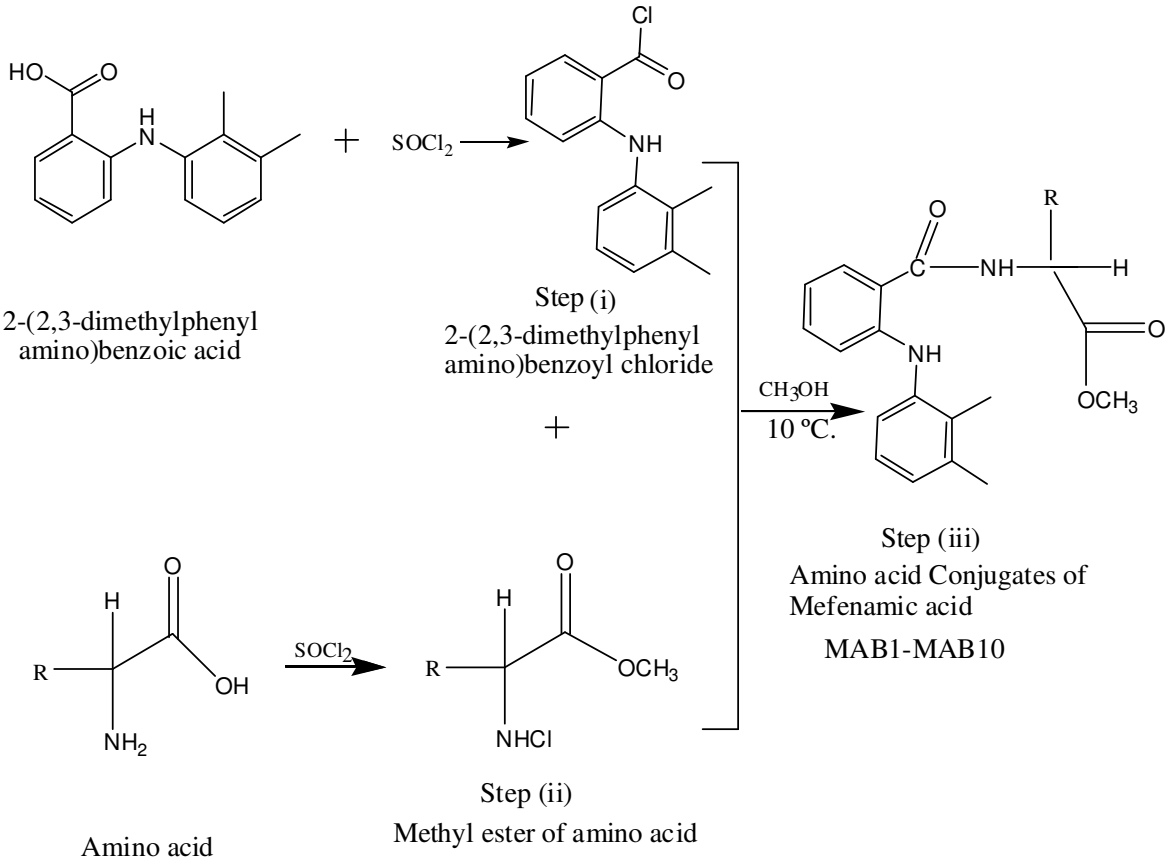
EXPERIMENTAL

^{1}H NMR spectra were recorded on Brucker-DRX (Japan) 300 MHz instrument for solution in dimethyl sulfoxide (DMSO). Mass spectra were recorded on Jeol SX-102 (Japan). All the solvents and chemicals were analytical grade.

Systhesis of amino acid conjugates of mefenamic acid

Step (1): Synthesis of mefenamic acid chloride: Mefenamic acid was dissolved in minimum amount of chloroform (5 mL) and thionyl chloride (3.2 mL) was slowly added to it. The mixture was refluxed for 10 h at 60 ºC temperature with continuous stirring on magnetic stirrer. The viscous liquid was immediately poured on petri dish and it was dry to give crude mefenamic acid chloride.

Step (2): Synthesis of methyl ester hydrochlorides of amino acids: Thionyl chloride (1.5 mL) was slowly added to methanol (4 mL) with cooling and amino acid in proportional amount was added to it. The mixture was refluxed for 6 h at 55 ºC with continuous stirring on magnetic stirrer. Excess thionyl chloride and solvent was removed under reduced pressure to produce crude amino acid methyl ester hydrochloride. The resulting solid product was collected and dried. It was re-crystallized from hot methanol. The crystals were collected after 24 h and washed twice with ether: methanol (4:6) mixture. The same procedure was followed to synthesize methyl ester hydrochlorides of all amino acids respectively.



Scheme-I for synthesis of amino acid conjugates of mefenamic acid

Step (3): Synthesis of amino acids conjugates of mefenamic acid: Ice cold, aqueous sodium hydroxide solution (5 %) was taken in 250 mL beaker and methyl ester of amino acid hydrochloride in proportional amount was added to it. The reaction mixture was mechanically stirred for 0.5 h at room temperature, beaker was transferred to an ice bath kept on mechanical stirrer, maintaining the temperature at 10 ºC. Mefenamic acid chloride was added in small portion with continuous stirring for 5 h. The solid that separated out was filtered off. The crude prodrug was re-crystallized from methanol. The same method was repeated to synthesize other amino acid conjugates of mefenamic acid **(Scheme-I)**.

Spectral data

Methyl 2-(2-(2,3-dimethylphenylamino)benzaamido-3-phenylpropanoate (MAB 1): Yield (39 %), m.p. 230-235 ºC m.w. (402.19) and R_f value (0.60). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9, (NH [*sec.* Amide]) 8, (CH_3) 2.30-3.67. Molecular peak in mass spectra 402.19 (100 %), 403.19, 404.19, 333.12, 304.15.

Methyl 2-(2-(2,3-dimethylphenylamino)benzaamido-3-mercaptopropanoate (MAB 2): Yield (41 %), m.p. 240-242 ºC, m.w. (358.45) and R_f value (0.80). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9 (NH [*sec.* amide]) 8, (CH_3) 2.30-3.67, (SH Thiol) 1.5. Molecular peak in mass spectra 358.14 (100 %), 359.14, 360.14, 312.21, 280.11, 213.12.

Methyl 2-(2-(2,3-dimethylphenylamino)benzaamido) acatate (MAB 3): Yield (30%), m.p. 212-215 ºC, m.w. 312.36 and R_f value (0.22). ^{1}H NMR Peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9 (NH [*sec.* amide]) 8, (CH_3) 2.30-3.67. Molecular peak in mass spectra 312.15 (100 %), 313.16, 314.15, 263.21, 253.

Methyl 6-amino-2-(2-(2,3-dimethylphenylamino) benzaamido)hexanoate (MAB 4): Yield (36 %), m.p. 284-290 ºC, m.w. 383.48 and R_f value (0.72). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9, (NH [*sec.* amide]) 8, (CH_3) 2.30-3.67. Molecular peak in mass spectra 383.22 (100 %), 384.22, 385.23, 314.22, 272.11, 199.23.

Methyl 2-(2-(2,3-dimethylphenylamino)benzaamido-5-guanidinopentanoate (MAB 5): Yield (35 %), m.p. 275-277 ºC, m.w. 422.53 and R_f value (0.54). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9 (NH [*sec.* amide]) 8, (CH_3) 2.30-3.67. (CH) 4.42. Molecular peak in mass spectra 411.23 (100 %), 412.23, 413.23, 263.24, 253, 238.34.

Methyl 2-(2-(2,3-dimethylphenylamino)benzaamido)-3-methylbutanoate (MAB 6): Yield (38 %) m.p. 295-297 ºC, m.w. 354.44 and R_f value (0.61). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9 (NH [*sec.* amide]) 8, (CH_3) 2.30-3.67. (CH) 4.42, (CH_3) 1.01. Molecular peak in mass spectra 339.17 (100 %), 340.17, 341.18, 284.18, 279.22, 217.12.

Methyl1-(2-(2,3-dimethylphenylamino)benzoyl) pyrrolidine-2-carboxylate (MAB 7): Yield (57 %), m.p. 250-255 ºC, molecular weight (352.48) and R_f value (0.60). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9 (NH [*sec.* amide]) 8, (CH_3) 2.30-3.67, (CH- in

pyrrolidine) 1.4-1.9, (CH-in pyrrolidine) 4.18. Molecular peak in mass spectra 352.18 (100 %), 353.16, 354.19, 301.12, 299.23.

Methyl 2-(2-(2,3-dimethylphenylamino)benzaamido)-3-hydroxypropanoate (MAB 8): Yield (30 %), m.p. 260-265 ºC, m.w. 342.39 and R_f value (0.88). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9 (NH [*sec.* amide]) 8, (CH_3) 2.30-3.67,(CH_2) 4.11-430, (OH) 1.99. Molecular peak in mass spectra 342.16 (100 %), 343.16, 344.16, 291.21, 284.23, 253.21.

Methyl 2-(2-(2,3-dimethylphenylamino)benzaamido) propanoate (MAB 9): Yield (30 %), m.p. 225-228 ºC, m.w. 326.39 and R_f value (0.72). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9 (NH [*sec.* amide]) 8, (CH_3 in aromatic ring) 2.30-3.67, (CH_3) 1.58. Molecular peak in mass spectra 326.16 (100 %), 327.17, 328.17, 288, 253, 212.11, 216.21.

Methyl 2-(2-(-2,3dimethylphenylamino)benzamido)-4(methylthio)butanoate (MAB 10): Yield 34 %, m.p. 230 - 235 ºC, m.w. 386.51 and R_f value (0.75). ^{1}H NMR peak in ppm (δ) (Ar-CH in benzene) 6.15-7.12 (Ar C-NH) 3.9 (NH [*sec.* amide]) 8, (CH_3 in aromatic ring) 2.30-2.67. Molecular peak in mass spectra 386.17 (100 %), 387.17 388.16, 312.11, 300.12, 262.11, 199.

Pharmacological evaluation

In vitro activity of synthesized conjugates

Inhibition of protein denaturation[6]: The reaction mixture (0.5 mL) was consisted of 0.45 mL bovine serum albumin (5 % aqueous solution) and 0.05 mL of synthesized conjugates, pH was adjusted to 6.3 using a 0.1 N HCl. The samples were heated at 57 ºC for 3 min. Mixture was allowed to cool and 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm. For control tests, 0.05 mL distilled water was used instead of conjugates, while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows:

$$\text{Percent inhibition} = \frac{100 - (\text{O.D. of test} - \text{O.D. of product control})}{\text{O.D. of control}} \times 100$$

where, O.D. = Optical density

The results were compared with mefenamic acid treated samples using Dunnet test, using graph pad 5 software.

Effect on membrane stabilization[6]: The reaction mixtures (4.5 mL) was consisted of 2 mL hypotonic saline (0.25 % NaCl), 1 mL of 0.15 M phosphate buffer (pH 7.4) and 1 mL solution of the synthesized conjugates in normal saline. 0.5 mL of 10 % rat RBC in normal saline was added. For control tests, 1 mL of isotonic saline was used instead of solution of the synthesized conjugates solution, while product control tests lacked red blood cells. The tubes were cooled under running tap water for 20 min. The mixtures were centrifuged and the absorbance of the supernatants measured at 560 nm. Per cent membrane stabilizing activity was calculated as follows:

$$\% \text{ Stabilization} = \frac{100 - (\text{O.D. of test} - \text{O.D. of product control})}{\text{O.D. of control}} \times 100$$

The results were compared with mefenamic acid treated samples using Dunnet test, using graph pad 5 software.

In-vivo activity

Analgesic activity (tail flick method)[7]: Analgesic activity of the synthesized prodrug was determined by tail flick method using thermal stimulus. A medicraft analgesiometer was used for the determination of pain threshold. Albino rats (180-200 g) were divided into twelve groups each comprising of three rats. The suspension of prodrug/drug compounds were prepared in distilled water using 1 % sodium carboxy methyl cellulose. Control received the same quantity of carboxy methyl cellulose with prodrug/drug. Prodrug, drug and control compounds were administered orally. Rats were placed into small restrain cages leaving the tail exposed. A light beam was focused (exerting radiant heat) to the proximal third of the tail. The rat tries to pull the tail away and rotates the head, this reaction known as escape reaction. The reaction time of this movement was recorded.

Inflammatory activity (paw edama)[7]: Antiinflammatory activity of synthesized prodrugs was determined by paw edema method utilizing carrageenan (0.1 mL, 1 %) as phlogistic agent. The animals used were albino rats. Rats (180-200 g) were divided into twelve groups, each comprising three rats including control, standard and treated group. The mefenamic acid and synthesized prodrugs (20 mg/kg) were administered orally in 1 % suspension of sodium carboxymethylcellulose. After 0.5 h of administration of mefenamic acid and synthesized prodrugs, carrageenan (0.1 mL, 1 % in normal saline) was injected into the planter surface of right paw of each animal as phlogistic agent. A varnier calliper was used to measure the volume of right paw of rats after 0.5, 1, 1.5 and 2 h.

Statistical analysis: Test of significance was analyzed by Dunnet test, using graph pad 5 software.

RESULTS AND DISCUSSION

The reactions of mefenamic acid chloride with methyl ester of amino acids were performed and conjugates of mefenamic acid with different amino acid *i.e*. phenylalanine, cysteine, glycine, lysine, arginine, valine, proline, serine, alanine and methionine were obtained (Table-1). The physical characterization data are given in Table-2. Structure of all the synthesized conjugates have been established based on their consistent ^{1}H NMR and mass spectral data. The synthesized conjugates showed the presence of amide and ester functional group along with the presence of aromatic and aliphatic ring, which was also evident in the ^{1}H NMR spectra of the synthesized conjugates.

The synthesized compounds were evaluated for their *in vitro* and *in vivo* analgesic and antiinflammatory activity. MAB1and MAB2 were showed equivalent analgesic and antiinflammatory activity compared to parent drug (Tables 3 and 4) respectively. MAB1and MAB2 also found to have equivalent activity as compared to parent drug in *in vitro* model (Table-5).

Conclusion

Based on the results obtained in the study, it may be concluded that conjugate approach can be successfully applied in attaining the goal of minimized gastrointestinal toxicity without loss of desired analgesic and antiinflammatory activities of the drug.

TABLE-1
SYNTHESIZED COMPOUND OF AMINO ACID CONGUGATES OF MEFENAMIC ACID

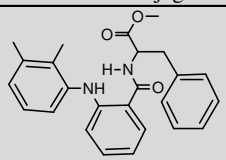
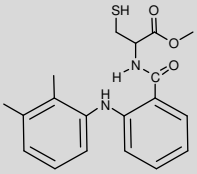
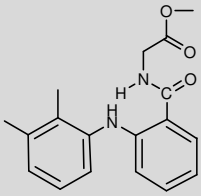
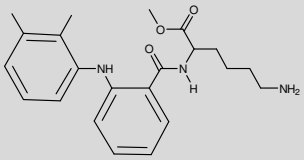
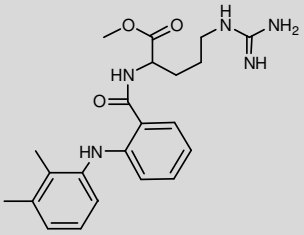
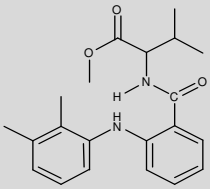
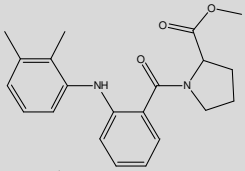
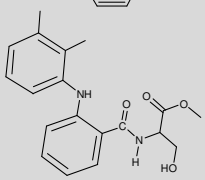
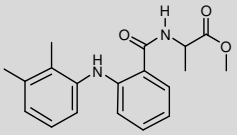
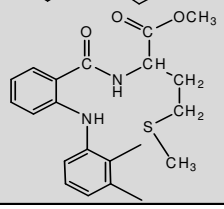
Compound code	Structure of conjugates	IUPAC name
MAB1		Methyl-2-[2-(2,3-dimethylphenylamino)-benzamido]-3-phenylpropanoate
MAB2		Methyl-2-[2-(2,3-dimethylphenylamino)-benzamido]-3-mercaptopropanoate
MAB3		Methyl-2-[2-(2,3-dimethylphenylamino)-benzamido]acatate
MAB4		Methyl-6-amino-2-[2-(2,3-dimethylphenyl-amino)benzamido]hexanoate
MAB5		Methyl-2-[2-(2,3-dimethylphenylamino)-benzamido]-5-guanidinopentanoate
MAB6		Methyl-2-[2-(2,3-dimethylphenylamino) benzamido]-3-methylbutanoate
MAB7		Methyl-1-[2-(2,3-dimethylphenylamino) benzoyl]pyrrolidine-2-carboxylate
MAB8		Methyl-2-[2-(2,3-dimethylphenylamino) benzamido]-3-hydroxypropanoate
MAB9		Methyl-2-[2-(2,3-dimethylphenylamino) benzamido]propanoate
MAB10		Methyl-2-[2-(2,3-dimethylphenylamino) benzamido]-4(methylthio)butanoate

TABLE-4
EFFECT OF SYNTHESIZED COMPOUNDS *IN-VIVO* ANTI-INFLAMMATORY ACTIVITY

Group no.	Compound code	Dose (mg/kg)	Swelling thickness (mm) ± SEM (% inhibition)			
			0.5 h	1 h	1.5 h	2 h
I	Control	-	0.41 ± 4.09	0.50 ± 4.15	0.57 ± 3.71	0.63 ± 4.20
II	Mefenamic acid	20	0.28 ± 4.29 (32.4)	0.33 ± 3.49 (39.3)**	0.34 ± 3.31 (39.7)**	0.34 ± 3.24 (45.7)***
III	MAB1	20	0.30 ± 2.50 (28.1)	0.32 ± 2.49 (36.4)**	0.35 ± 2.71 (37.4)**	0.37 ± 2.18 (40.9)***
IV	MAB2	20	0.29 ± 3.35 (29.7)	0.31 ± 2.49 (37.8)**	0.35 ± 2.38 (38.6)**	0.36 ± 2.42 (39)***
V	MAB3	20	0.35 ± 4.62 (14.1)	0.41 ± 4.61 (18.3)	0.44 ± 4.79 (21.3)	0.49 ± 4.69 (21.6)
VI	MAB4	20	0.26 ± 3.36 (36.4)	0.32 ± 3.82 (34.7)**	0.36 ± 3.33 (36.3)**	0.42 ± 3.39 (33.2)**
VII	MAB5	20	0.34 ± 4.94 (17.3)	0.37 ± 5.33 (25)	0.39 ± 3.43 (30.6)*	0.42 ± 3.12 (33)**
VIII	MAB6	20	0.35 ± 5.12 (14)	0.39 ± 5.38 (22.1)	42.3 ± 5.55 (26)	0.44 ± 5.04 (28.9)
IX	MAB7	20	0.31 ± 3.49 (23.9)	0.34 ± 3.15 (32)	0.43 ± 5.48 (24.5)	0.41 ± 3.75 (34.6)**
X	MAB8	20	0.33 ± 3.66 (19.5)	0.39 ± 4.49 (22)	0.43 ± 3.71 (24)	0.47 ± 4.o7 (23.3)
XI	MAB9	20	0.29 ± 3.08 (28.3)	0.35 ± 3.21 (28.5)	0.41 ± 2.72 (27.9)*	0.46 ± 2.64 (26.2)*
XII	MAB10	20	0.35 ± 2.13 (15.5)	0.40 ± 2.18 (18.8)	0.46 ± 1.85 (18.9)	0.51 ± 1.91 (18)

Where, *P < 0.05, **P < 0.01, ***P < 0.001; MAB1- MAB 10 synthesized compounds code

TABLE-2
PHYSIOCHEMICAL PROPERTY OF SYNTHESIZED COMPOUNDS

Compound code	m.w.	Yield (%)	m.p. (ºC)	R_f value
MAB 1	402.19	39	230-235	0.60
MAB 2	358.45	41	240-242	0.80
MAB 3	312.36	30	212-215	0.22
MAB 4	383.48	36	284-290	0.72
MAB 5	411.53	35	275-277	0.54
MAB 6	354.44	38	295-297	0.61
MAB 7	352.48	57	250-255	0.68
MAB 8	342.39	30	260-265	0.88
MAB 9	326.39	30	225-228	0.72
MAB 10	386.51	34	222-225	0.75

TABLE-3
EFFECT OF SYNTHESIZED COMPOUNDS *IN-VIVO* ANALGESIC ACTIVITY

Group no.	Compound code	Dose (mg/kg)	Increase in reaction time (%)
I	Control	-	-
II	Mefenamic acid	20	56.67
III	MAB1	20	51.32
IV	MAB2	20	53.87
V	MAB3	20	23.23
VI	MAB4	20	18.33
VII	MAB5	20	23.23
VIII	MAB6	20	31.67
IX	MAB7	20	23.45
X	MAB8	20	18.57
XI	MAB9	20	25.34
XII	MAB10	20	26.34

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TABLE-5
EFFECT OF SYNTHESIZED COMPOUNDS *IN-VITRO* (PROTEIN DENATURATION AND MEMBRANE STABILITIZATION) MODEL

Compound code	Protein denaturation (%) mean ± SD	Membrane stabilitization (%) mean ± SD
MAB1	50.74 ± 0.65	52.26 ± 0.22
MAB2	28.45 ± 0.39	37.35 ± 0.31
MAB3	23.62 ± 0.57	20.31 ± 0.54
MAB4	24.83 ± 0.76	14.11 ± 0.14
MAB5	20.03 ± 0.07	16.17 ± 0.17
MAB6	23.41 ± 0.38	22.58 ± 0.52
MAB7	33.45 ± 0.39	23.52 ± 0.45
MAB8	18.41 ± 0.38	29.25 ± 0.26
MAB9	28.52 ± 0.41	26.39 ± 0.23
MAB10	28.48 ± 0.41	20.43 ± 0.43
Mefenamic acid	51.15 ± 0.13	60.78 ± 0.67

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