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EVALUATION OF NOVEL L-ARGINGNE ANALOGS FOR ANTIINFLAMMATORY AND RELATED ACTIVITIES

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ABSTRACT

The role of nitric oxide (NO) in inflammation represents one of the most studied yet controversial subjects in physiology. A number of reports have been demonstrated that NO possesses potent anti-inflammatory properties, whereas an equally impressive number of studies suggest that NO induces cell and tissue dysfunction thereby promoting inflammation. It is known for a long time that inflammatory processes are associated with enhanced production of a number of substances, such as Bradykinin, IL-β, TNF-α, Substance P, PGE2, Interferon-gamma (INF-γ), Nerve growth factor (NGF), Serotonin and Histamine. Practically all of these substances, some of which are produced by neurons, have shown to be associated with enhanced production of NO. Inhibitors of NO biosynthesis may provide a novel therapeutic approach for various diseases. In addition, design of selective inhibitors of NOS may act as useful tools for investigating other biological functions of NO.

KEYWORDS

Nitric oxide, Bradykinin, IL-β, TNF-α, Nerve growth factor, Serotonin and Histamine.

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INTRODUCTION[1,2]

NO is a key inter and intra molecular messenger synthesized by NOS from L-arginine and molecular oxygen, which is involved in a number of physiological and pathological process in mammalians i.e., maintenance of vascular tone, neuronal signaling and host response to infection. Three structurally distinct isoforms of NOS have been identified:

Neuronal (nNOS)-Type-I
Endothelial (eNOS)-Type-III
Inducible (iNOS)-Type-II

NO is synthesized enzymatically from L-arginine via a five electron transfer process in numerous tissues and cell types by these three distinct isoforms of the enzymes, NOS. Two of these isoforms are constitutive, predominant in the vascular endothelium (eNOS Type-III) and in the nervous system (nNOS Type-I). Under normal physiological conditions, these constitutive forms of NOS generate low transient levels of NO (picomolar to nanomolar concentrations) in response to increase in intra cellular concentration. These low levels of NO act to regulate blood pressure, platelet adhesion, gastrointestinal motility, bronchomotor tone and neurotransmission[3].

The expression of the third isoform (iNOS Type-II) is induced by endotoxin and /or cytokines and generates high sustained levels of NO (up to micromolar concentrations). These elevated levels of NO and resulting NO derived metabolites cause cellular toxicity and tissue damage and is thought to contribute to the pathophysiology of a number of human diseases viz., inflammation, rheumatoid arthritis and septic shock). Although nNOS mediates several physiological functions, over production of NO by nNOS has also been reported in a number of clinical disorders including convulsions, pain, schizophrenia and neurodegenerative disorders like Alzheimer's and Parkinson's[4].

Lipids are the most susceptible targets for the free radical attack. NO because of its free radical nature, though not too reactive, reacts rapidly with superoxide and damages cell by producing extremely reactive peroxynitrite and its conjugate acid (ONOOH-) which are the major sources for lipid peroxidation and cellular damage. In biological membranes extensive lipid peroxidations causes alteration in fluidity, fall in membrane potential, increases permeability to ions and eventually rupture leading to release of cell organelles such as lysosomal hydrolytic enzymes (Figure No.1).

Formation

L-arginine nNOS, eNOS and iNOS $\longrightarrow$ L-Citrulline + NO.

Decomposition

NO - Degrades rapidly $\longrightarrow$ NO_2^-, NO_3^- .

The objectives of the present work is

- To evaluate anti inflammatory activity of the novel L-arginine analogs by Rat paw oedema method.
- To evaluate the analgesic activity by writhing test in mice.
- To evaluate the antipyretic activity by using antipyretic testing in rats.
- To evaluate the *in vitro* anti-oxidant activity of those compounds that exhibited high % activity.

MATERIAL AND METHODS[5,6]

List of chemicals:

- Carrageenan-Sigma
- Brewer's yeast-local market
- Glacial acetic acid-Sd fine
- Ibuprofen, Paracetamol-Dr.Reddy's laboratories
- Thiobarbituric acid (TBA)-Sd fine
- Trichloro acetic acid (TCA)-Sd fine
- Sodium nitroprusside-Sd fine
- N-napthyl ethylenediamine dihydrochloride-Sd fine
- 1-1-diphenyl 1-2-picrylhydrazyl (DPPH)-Sigma.

Griess reagent

- Sulphanilamide:4 g
- N-napthyl ethylene diamine:0.2 g
- 10% orthophosphoric acid: 10 ml
- Diluted up to 100 ml with distilled water.

Instruments

- Systronic UV-VIS-spectrophotometer.
- REMI centrifuge machine.
- pH meter.

Experimental animals[7]

Male albino rats weighing (120-150 g) and male albino mice weighing (18-25 g) were used for the evaluation of pharmacological activities. They were kept in polypropylene cages at 25+ 2o C, with relative humidity of 45-55% under 12 hours light and dark cycles. All the animals were acclimatized

to the laboratory conditions for a week before use. They were fed with standard animal feed (Lipton India) and water ad libitum.

Acute toxicity studies[8]

Acute oral toxicity study was performed as per OECD-423 guide lines (acute toxic class method), (Ecobichon, 1997). Male albino rats (n=6) were selected by random sampling techniques were used for acute toxicity study. The animals were subjected to fasting overnight providing only water, after which the drugs were administered orally at dose of 5mg/kg body weight by gastric intubation and observed for 14 days. If mortality was observed in 2 out of 3 animals, then the dose administered was assigned as toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 300 and 2000mg/kg body weight.

Anti inflammatory activity

Paw edema method[9,10]

Paw edema was induced by injecting 0.1 ml of 1% carrageenan in physiological saline into the subplantar tissues of the left hind paw of each rat.

1% carrageenan solutions were prepared 24 hours before the experiment. Male albino rats weighing 150-180 g were divided into groups of 6 animals each and subjected to fasting over night. The control, standard and the test drugs at a dose of 100mg/kg body weight were administered to the animals 1 hour before carrageenan could be injected into the hind paw of the animals. One hour after oral administration the rats were challenged by an intraplantar injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hind paw. The paw was marked with ink at the level of the lateral malleous and immersed into the instrument i.e., the plethysmometer the paw volume was measured immediately after intraplantar injection of carrageenan.

From then on graded increase in the paw volume was noted at regular intervals of 30, 60, 120 and 180 minutes. The increase in paw volume in the test group is compared to that of the standard and control.

Analgesic activity

Writhing test in mice

Pain is induced by injecting irritants into the peritoneal cavity of mice. 0.1 ml of a 0.6% solution of glacial acetic acid is injected intraperitoneally to mice with a weight between 18-25 g.

Test animals were divided into groups of 6 animals each and subjected to fasting over night. Test or standard drugs were administered at various pretreatment times prior to acetic acid administration. The mice are placed individually in a glass beaker and five minutes are allowed to elapse. The mice were then observed for a period of ten minutes and the numbers of wriths were recorded for each animal. For scoring purposes, a writh is indicated by stretching of the abdomen with simultaneous stretching of at least one hind limb. The formula for computing % inhibition is as follows:

% inhibition = avg., writhes in control – avg., writhes in test X 100

Writhes in control

Those drugs which inhibit writhing more than 70% were considered to be good activity and those with less than 70% inhibition were considered to have minimal activity.

Antipyretic activity[11-13]

Testing in rats

The subcutaneous injection of Brewer's yeast suspension is known to produce fever in rats.

A 15% suspension of Brewer's yeast in 0.9% saline was prepared. Groups of 6 rats each were used. The temperatures were recorded by insertion of a thermocouple to a depth of 2cm into the rectum. The initial temperatures were recorded. The animals were fevered by injection of 10 ml/kg of Brewer's yeast suspension simultaneously in the back below the nape of the neck. Immediately after administration food is withdrawn 18 hours post challenge. The rise in temperature was recorded the animals with a body temperature of at least 38o C were taken into the test. The test and control were administered orally. Rectal temperatures were recorded at 30, 60, 120 and 180 minutes post dosing.

***In vitro* Antioxidant studies**

Assay for NO scavenging activity

Sodium Nitroprusside (10 µM) in phosphate buffer pH 7.7 was incubated with 25, 50, 75, 100 and 125 µM concentrations of drug dissolved in a suitable solvent (Dixon/methanol) and tubes were incubated at 25 o C for 120 minutes. At intervals, 0.5ml of incubation solution was removed and diluted with 0.5ml of Griess reagent. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent N-napthyl ethylenediamine was measured at 546nm.

Interaction with stable free radical DPPH

This assay is based on the measurement of the scavenging ability of antioxidant test substances towards the stable radical. The free radical scavenging activity of the drugs was examined *in vitro* using DPPH radical. Solutions of various drugs at different concentrations of 25, 50, 75, 100 and 125 µM were added to 100 µM DPPH in ethanol and tubes were kept at an ambient temperatures for 20 minutes and absorbance was measured at 517 nm. Positive control was used. Results were expressed as means of triplicates.

Statistical analysis

All the data were represented as means ±SEM. Statistical analysis was performed by ANOVA test for multiple comparisons followed by Bonferroni's test. The statistical significance was set accordingly.

RESULTS

All results are showed in Table No-1-5 and Figure No.2-9.

Table No.1: Anti inflammatory activity of L-arginine analogs (peptides)

S.No	Compound	Dose (mg/kg)	Time (h)	paw volume (ml) mean±SEM	% inhibition	Significance
1	Control	---	1 2 3	0.31±0.077 0.51±0.065 0.74±0.041	---	---
2	Ibuprofen	100	1 2 3	0.125±0.004 0.10±0.013 0.06±0.013.	59.6 86.23 88.48	p<0.001
3	2	100	1 2 3	0.20±0.023 0.32±0.037 0.49±0.068	35.48 37.25 23.78	NS
4	3	100	1 2 3	0.39±0.065 0.19±0.041 0.30±0.120	23.52 38.7 59.45	p<0.001
5	4	100	1 2 3	0.27±0.053 0.42±0.054 0.52±0.053	59.6 86.23 88.48	p<0.001
6	5	100	1 2 3	0.29±.060 0.38±.058 0.52±.050	6.4 25.4 29.7	NS
7	7	100	1 2 3	0.19±0.056 0.32±0.072 0.51±0.029	38.7 37.25 31.08	p<0.001
8	9	100	1 2 3	0.32±.039 0.19±.048 0.38±.043	37.25 38.7 48.65	p<0.001
9	11	100	1 2 3	0.29±.005 0.125±.05 0.07±.031	63.14 75.4 77.4	p<0.001

NS: Not significant

Table No.2: Anti inflammatory activity of L-arginine analogs (imidazoles)

S.No	Compound	Dose (mg/kg)	Time (h)	paw volume (ml) mean±SEM	% inhibition	Significance
1	Control	---	1 2 3	0.31±0.077 0.51±0.065 0.74±0.041	---	---
2	Ibuprofen	100	1 2 3	0.125±0.004 0.10±0.013 0.06±0.013.	59.6 86.23 88.48	p<0.001
3	14	100	1 2 3	0.06±.013 0.26±.101 0.46±.087	79 48.03 37.8	NS
4	15	100	1 2 3	0.39±.092 0.115±.032 0.08±.034	47.29 77.45 72.5	p<0.001
5	16	100	1 2 3	0.047±.020 0.15±.031 0.45±.094	84.83 70.58 39.18	NS
6	17	100	1 2 3	0.09±.005 0.25±.050 0.46±.025	70.96 50.98 37.8	p<0.001
7	20	100	1 2 3	0.09±.021 0.20±.016 0.36±.069	70.96 60.78 51.35	p<0.001
8	21	100	1 2 3	0.017±.008 0.07±.014 0.16±.025	94.5 86.27 78.37	p<0.001

NS: Not significant

Table No.3: Analgesic activity of L-arginine analogs

S.No	Compound	Dose (mg/kg)	No. of wriths (mean±SEM)	% Inhibition	Significance
1	Control	---	66.33±2.29	---	---
2	Ibuprofen	100	37.33±2.41	43.72	p<0.001
3	3	100	29.33±1.82	55.78	p<0.001
4	9	100	38.33±1.86	41.4	p<.001
5	11	100	52.33±3.93	21.1	NS
6	15	100	42±2.29	36.68	p<.001
7	20	100	48.5±1.74	26.8	p<.001
8	21	100	45.16±3.63	31.9	NS

Table No.4: *In vitro* free radical scavenging effect of L-arginine analogs by nitric oxide scavenging method

S.No	Compounds	Concentration (µM) % scavenging (Mean± SEM) of triplicates				
		25	50	75	100	125
1	**Standard**	79.11±0.246*	82.45±0.103*	84.67±0.262*	91.95±0.108*	96.49±0.088*
2	3	13.82±0.352*	15.54±0.456*	24.65±0.410*	33.44±0.080*	44.69±0.205*
3	9	12.66±0.669*	22.69±0.726*	34.36±0.046*	35.94±0.456*	39.18±0.563*
4	11	66.26±0.786*	69.64±0.372*	85.86±0.462*	87.48±0.163*	92.9±0.317*
5	15	24.3±0.104*	36.0±0.489*	49.87±0.220*	70.99±0.752*	72.26±0.182*
6	20	2.17±0.430*	19.36±0.363*	21.47±0.270*	29.54±0.078*	39.13±0.285*
7	21	19.12±0.390*	28.75±0.155*	38.19±0.177*	41.48±0.275*	43.13±0.158*

*** (P<0.001) when compared to control**

Table No.5: *In vitro* free radical scavenging effect of L-arginine analogs by DPPH method

S.No	Compound	Concentration (µM) % scavenging (Mean± SEM) of triplicates				
		25	**50**	**75**	**100**	**125**
1	Standard	78.40±0.246*	81.06±0.208*	85.15±0.205*	89.61±0.192*	93.35±0.198*
2	3	1.6±0.209*	4.4±0.061*	11.4±0.186*	20.55±0.096*	35.09±0.225*
3	9	7.58±0.309*	13.84±0.153*	18.07±0.061*	24.48±0.086*	39.65±0.225*
4	11	31.04±0.052*	42.4±0.148*	44.6±0.145*	59.39±0.059*	66.7±0.242*
5	15	39.9±0.245*	44.43±0.081*	63.10±0.081*	68.39±0.089*	77.84±0.083*
6	20	15.23±0.376*	21.33±0.0.131*	29.57±0.001*	35.55±0.086*	42.83±0.044*
7	21	32.84±0.0.292*	44.54±0.144*	53.54±0.196*	61.64±0.065*	64.9±0.068*

*** (P<0.001) when compared to control**

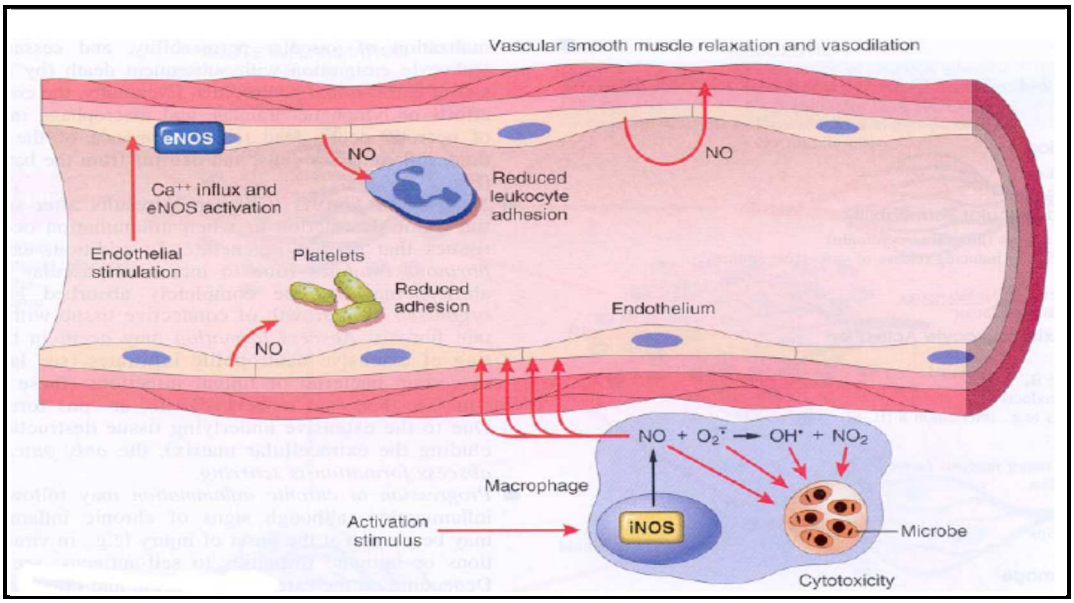
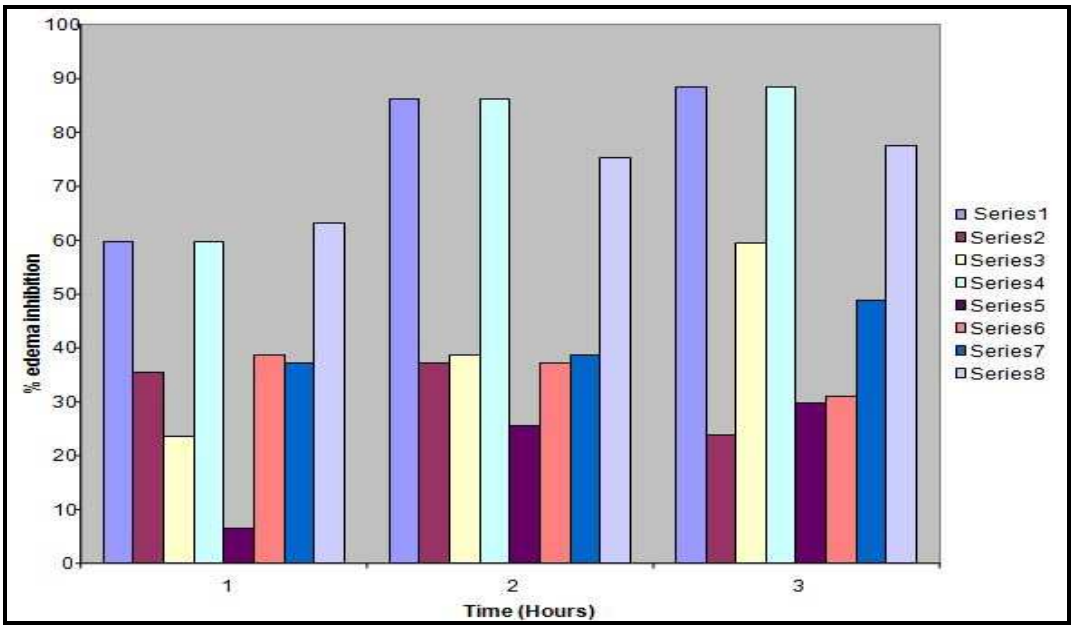
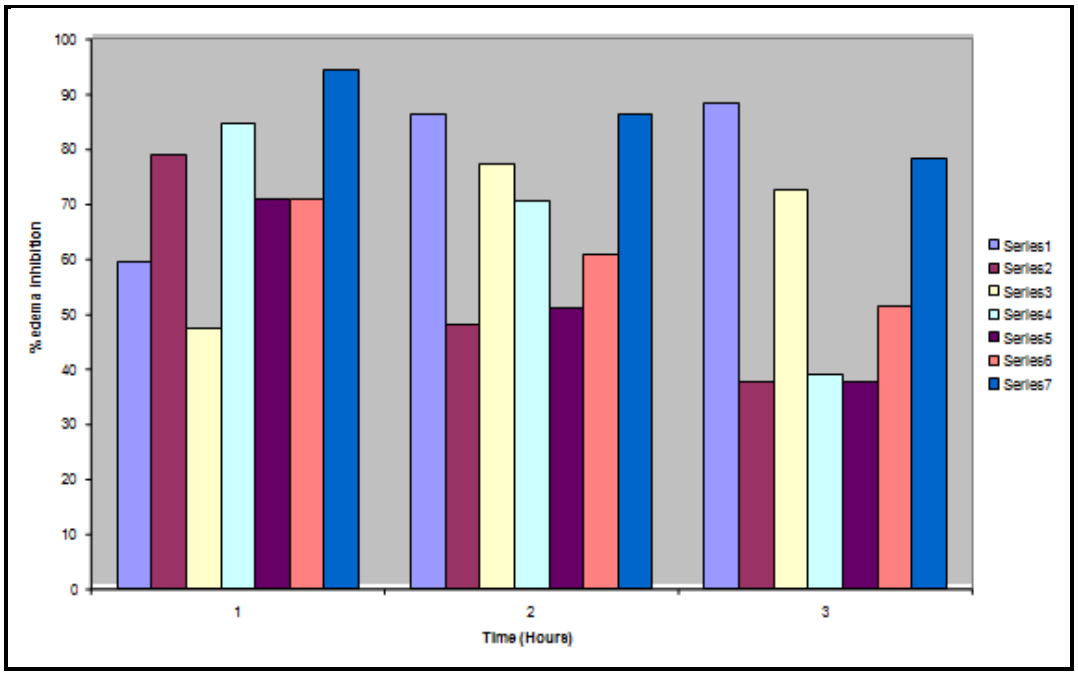


Figure No.1: Formation, decomposition and reactivity of NO



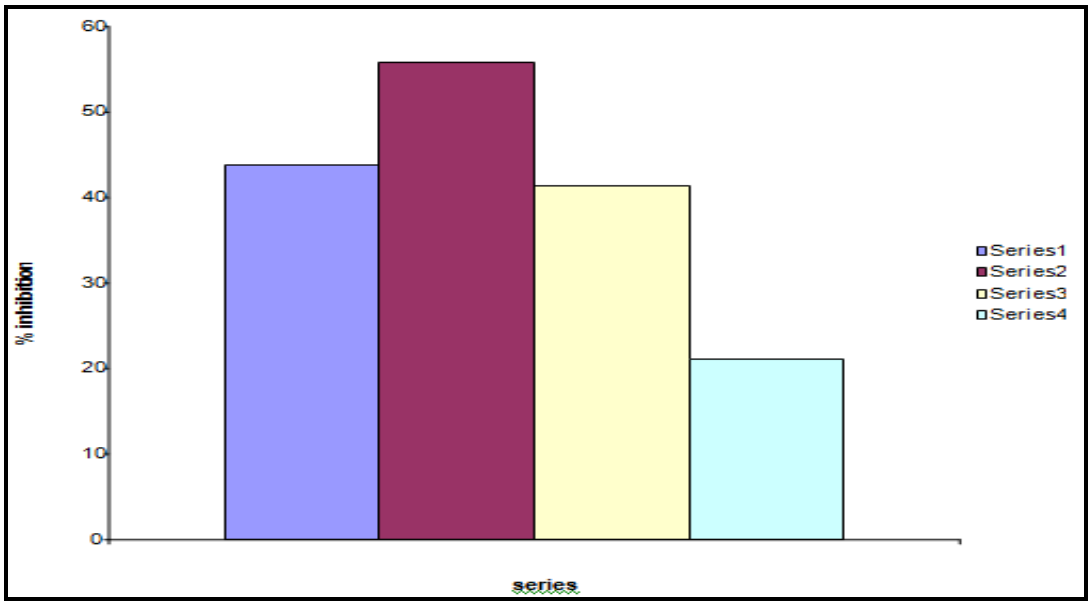
Series1: Standard; Series2: Compound-2; Series3: Compound-3; Series4: Compound-4; Series5: Compound-5; Series6: Compound-7; Series7: Compound-9; Series8: Compound-11.

Figure No.2: Anti inflammatory activity of L-arginine analogs (peptides)



Series1: Standard; Series2: Compound-14; Series3: Compound-15; Series4: Compouns-16; Series5: Compound-17; Series6: Compound-20; Series7: Compound-21.

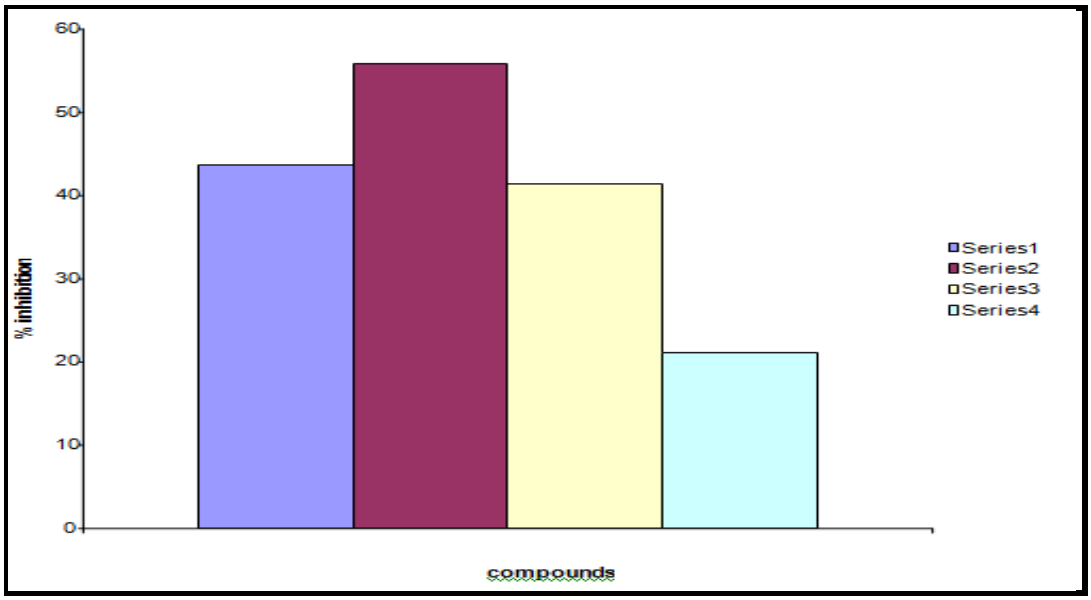
Figure No.3: Anti inflammatory activity of L-arginine analogs (imidazoles)



Series1: Standard; Series2: Compound-3; Series3: Compound-9;

Series4: Compound-11.

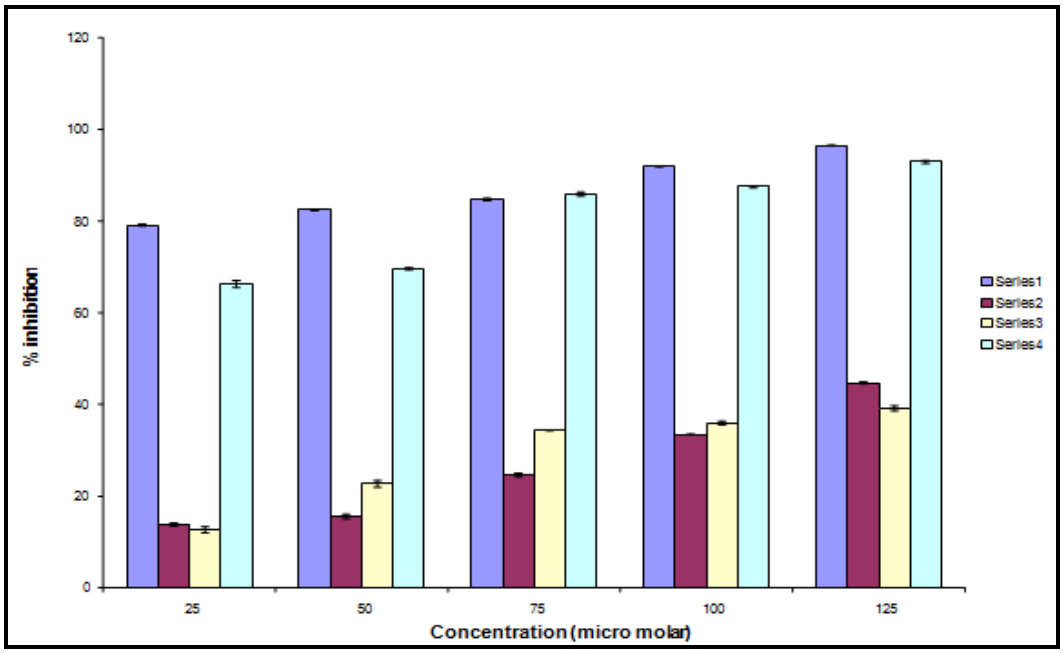
Figure No.4: Analgesic activity of L-arginine analogs (peptides)



Series1: Standard; Series2: Compound-3; Series3: Compound-9;

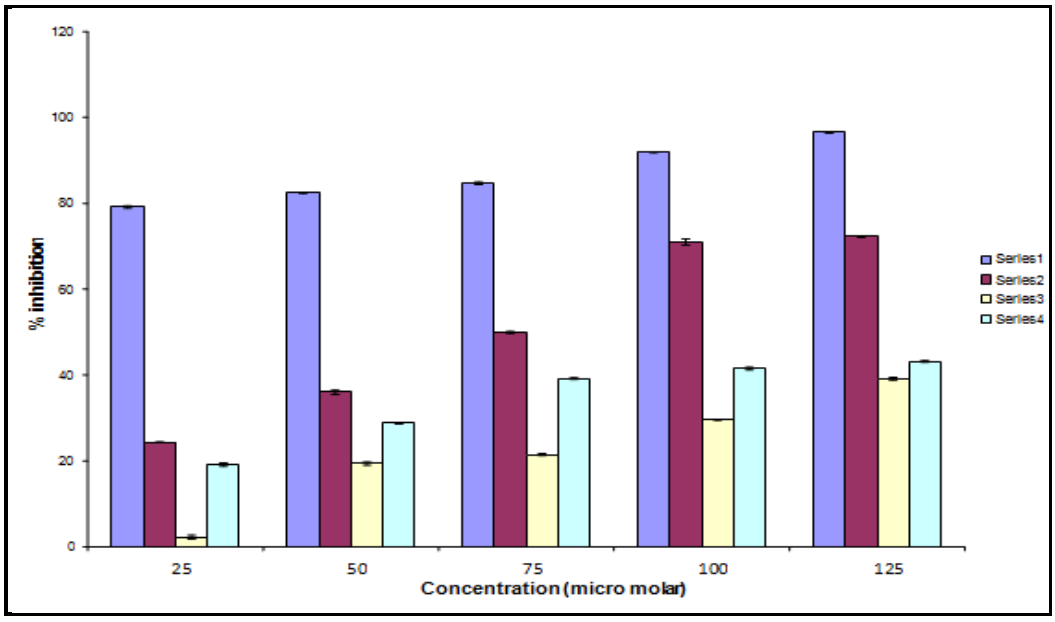
Series4: Compound-11.

Figure No.5: Analgesic activity of L-arginine analogs (imidazoles)



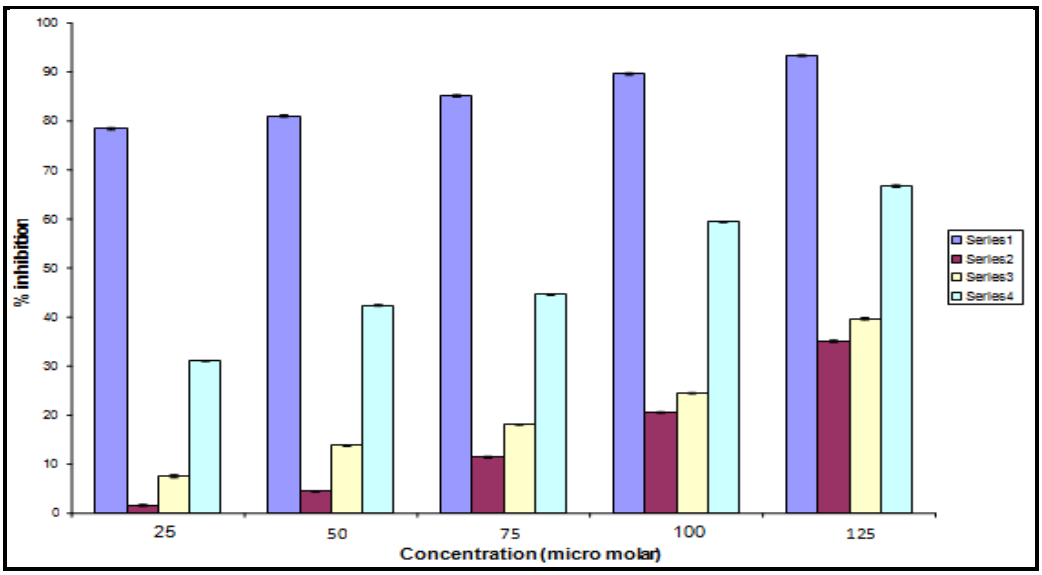
Series1: Standard; Series2: Compound-3; Series3: Compound-9; Series4: Compound-11

Figure No.6: *In vitro* free radical scavenging effect of L-arginine analogs by nitric oxide scavenging method (peptides)



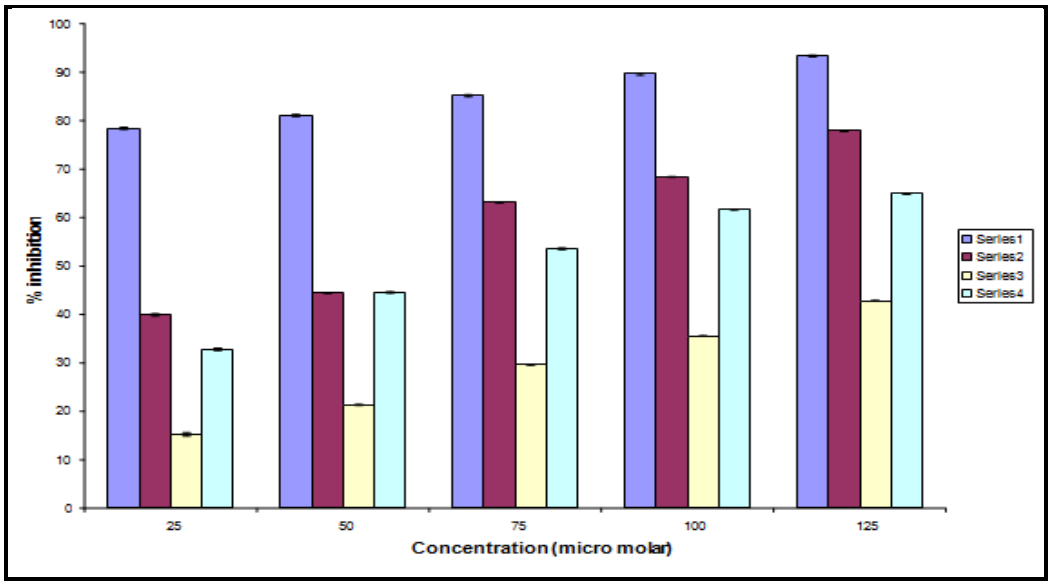
Sries1: Standard; Series2: Compound-15; Series3: Compound-20; Series4: Compound-21

Figure No.7: *In vitro* free radical scavenging effect of L-arginine analogs by nitric oxide scavenging method (imidazoles)



Series1: Standard; Series2: Compound-3; Series3: Compound-9; Series4: Compound-11

Figure No.8: *In vitro* free radical scavenging effect of L-arginine analogs by DPPH method (peptides)



Sries1: Standard; Series2: Compound-15; Series3: Compound-20; Series4: Compound-21

Figure No.9: *In vitro* free radical scavenging effect of L-arginine analogs by DPPH method (imidazoles)

CONCLUSION

In conclusion, the present work was carried out to evaluate the L-arginine analogues as potent anti-inflammatory and analgesic agents with antioxidant properties by inhibiting NOS. Effects of these compounds could be further studied by employing other models of acute and chronic inflammation, the ulcerogenic potential of these compounds can also be studied. Enzyme specificity of these compounds can be studied by employing radio ligand binding studies.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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