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EVALUATION OF *IN-VITRO* ANTI-OXIDANT ACTIVITY OF SOME SYNTHESIZED NOVEL L-ARGININE ANALOGUES

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ABSTRACT: A series of novel L-arginine analogues were synthesized and screened for in-vitro anti-oxidant activity by Nitric oxide (NO) scavenging and 1-1-diphenyl-2-picrylhydrazyl (DPPH) scavenging methods. The purity of the synthesized compounds has been characterized by various analytical techniques such as UV, FTIR and TLC. The synthesized compounds were evaluated for *in-vitro* anti-oxidant activity by NO scavenging and DPPH scavenging methods. The study concluded that the compounds4, 5, 6, 7, 8, 10, 11 exhibited very significant anti-oxidant action in NO scavenging method and compounds 4, 6, 7, 8, 10, 11 has exhibited significant anti-oxidant activity in DPPH scavenging method when compared with that of the standard Ascorbic acid.

INTRODUCTION: Many present day diseases are reported to be an impaired imbalance of the pre-oxidant anti-oxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either on account of increased generation of free radicals caused by excessive oxidant stress or due to poor scavenging in the body caused by depletion of the dietary antioxidants. Reactive oxygen species differ significantly in their interactions and can cause extensive cellular damage such as nucleic acid strand scission, modification of polypeptides, lipid peroxidation etc[1].

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Free radicals of different forms are constantly generated for specific metabolic requirements and quenched by an efficient anti-oxidant network in the body. When the generation of these species exceeds the levels of anti-oxidant mechanism, it leads to oxidant damage of tissues and bio molecules, eventually leading to disease conditions, especially degenerative diseases[2]. Anti-oxidants can be defined as "any substance which significantly delays or inhibits oxidative damage to a target molecule".

Anti-oxidants are the first line of defense against free radical damage and are critical for maintaining optimum health. The need for anti-oxidants becomes even more critical with increased exposure to free radicals. As part of a healthy lifestyle and a well-balanced, wholesome diet, anti-oxidant supplementation is now being recognized as an important means of improving free radical protection[3].The recognition of NO production by

activated macrophages as part of the inflammatory process was an important milestone for assessing both biological production and the phenomenon of induction of Nitric oxide synthase activity. NO may mediate inflammation andcontribute to cell death by acting directly on transcriptional factors[4]. Identification of N-methyl-L-Arginine (L-NMA) as the first inhibitor of NO biosynthesis led to the design of selective iNOS inhibitors[5]. Hence the present study was planned to synthesize some novel substituted L-arginine analogues and to evaluate for anti-oxidant activity.

MATERIALS AND METHODS:

Synthetic Chemistry

STEP-1 (Synthesis of 4-benzylidine-2-phenyl oxazole – 5- ones):

A mixture of benzoyl glycine, redistilled benzaldehyde, acetic acid and anhydrous sodium acetate was heated on an electric hot plate with stirring. On liquefaction it was heated for 2hrs and ethanol was added slowly and the mixture was allowed to stand overnight. The product obtained is washed with boiling water and dried at 100^{o}C. The product obtained in step-I was used in step-2 for further synthesis.

STEP-II (Synthesis of substituted L-arginine analogues):

The product obtained in step-I was reacted with unsubstituted L-Arginine and some substituted L-Arginine in alkali like NaOH and acetone which results in clear solution after 2-3hrs of reaction. The solution thus obtained was acidified by the addition of HCl. The products separated were unsubstituted and some substituted L-arginine analogues. L-Arginine analogues were washed with cold water and dried. The compounds thus obtained were used for screening anti-oxidant activity after purification and characterization. The %yield, melting points, Rf values and molecular formula of various substituted L-arginine analogues are tabulated in **Table 1.** The synthesized compounds were further purified by recrystallization and characterized by UV, TLC, FT-IR spectroscopy.

TABLE: 1-PHYSICAL DATA OF SUBSTITUTED L-ARGININE ANALOGUES (1-11)

Compound Name	R	Melting Point (^{o}C)	Rf value	% yield	Molecular Formula
1	H	205	0.62	76	$C_{22}H_{25}N_5O_4$
2	4-Cl	180 – 185	0.76	66	$C_{22}H_{24}N_5O_4Cl$
3	4-OCH_3	210	0.48	65	$C_{23}H_{27}N_5O_5$
4	4-OH	190	0.66	45	$C_{22}H_{25}N_5O_5$
5	4-OH, 3-OCH_3	175 – 177	0.82	47	$C_{23}H_{27}N_5O_6$
6	5-Br, 4-OH, 3-OCH_3	170 – 172	0.72	46	$C_{23}H_{26}N_5O_6Br$
7	4-$N(CH_3)_2$	198 – 200	0.56	79	$C_{24}H_{30}N_6O_4$
8	4–$(CH_3)_2$	190	0.692	55	$C_{25}H_{31}N_5O_4$
9	4-NO_2	195	0.833	51	$C_{22}H_{24}N_6O_6$
10	4-CH_3	205	0.44	53	$C_{23}H_{27}N_5O_4$
11	5-I, 4-OH, 3-OCH_3	207	0.51	45	$C_{23}H_{26}N_5O_6I$

ANTI-OXIDANT ACTIVITY:

Nitric oxide scavenging method

Nitric oxide will be generated by sodium nitroprusside in solution. In the presence of an anti-oxidant or nitric oxide scavenger the amount of no generated will be less. The excess NO will be estimated by Griess reagent which is a mixture of sulphanilic acid and naphthyl ethylene diamine dihydrochloride. The nitric oxide will give pink color complex estimated at 540nm.

Procedure: To a reaction mixture (6ml) containing sodium nitroprusside (10mM, 4ml), phosphate buffer saline (PBS, 1.0ml) and 1.0ml of different concentration of test compounds and standard were incubated at 25^{o}C for 150minutes. After incubation, 0.5ml of the reaction mixture containing nitrate was removed and 1.0ml of sulphanilic acid was added, mixed well and allowed to stand for 5min for completion of diazotization. Then 1.0ml naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30min in a dark room temperature. The absorbance of these solutions was measured at 540nm against corresponding blank solution without sodium nitroprusside[6]. Plotting the percentage NO

scavenging against concentration gave the standard curve and the percentage scavenging was calculated from the following equation:

% scavenging = Absorbance of blank – Absorbance of test/ Absorbance of blank X 100.

IC_{50} was obtained from a plot between concentration of test compounds and % scavenging. Ascorbic acid was used as standard for comparison. The results are tabulated in **Table 1.**

DPPH scavenging method

DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electron, the methanolic solution of DPPH shows a strong absorption band at 517nm. DPPH radical reacts with various electron donating molecules (reducing agents or anti-oxidants). When electrons become paired off, bleaching of the DPPH solution is the result. This results in the formation of the colorless 2, 2-diphenyl -1-picrylhydrazine. Reduction of the DPPH radicals can be estimated quantitatively by measuring the decrease in absorbance at 517nm.

Procedure: Equal volumes of 100µM 2, 2-diphenyl -1-picrylhydrazyl in methanol was added to different concentrations of test compounds (100 µM/ml) in methanol, mixed well and kept in dark for 20min. The absorbance at 517nm was measured using the spectrophotometer UV-1650, Shimadzu[7].The % scavenging and IC_{50} values were determined as explained in NO scavenging method. The results are tabulated in **Table 2.**

TABLE: 1 *IN –VITRO* FREE RADICAL SCAVENGING EFFECT OF L-ARGININE ANALOGUES BY NITRIC OXIDE SCAVENGING METHOD

Compound	Concentration (µM)				
	% scavenging (Mean ± SEM)				
	25	50	75	100	125
Standard (Ascorbic acid)	79.11 ± 0.246*	82.45 ± 0.103*	84.67 ± 0.262*	91.95 ± 0.108*	96.49 ± 0.088*
Compound 1	15.76 ± 0.356*	19.67 ± 0.145*	27.98 ± 0.456*	38.98 ± 0.123*	56.76 ± 0.087*
Compound 2	23.78 ± 0.456*	35.65 ± 0.132*	46.67 ± 0.246*	49.76 ± 0.278*	53.78 ± 0.104*
Compound 3	13.82 ± 0.352*	15.54 ± 0.456*	24.65 ± 0.410*	33.44 ± 0.080*	44.69 ± 0.205*
Compound 4	35.78 ± 0.346*	47.87 ± 0.234*	61.23 ± 0.345*	69.76 ± 0.008*	75.98 ± 0.265*
Compound 5	43.78 ± 0.780*	54.64 ± 0.234*	68.98 ± 0.023*	82.67 ± 0.107*	93.76 ± 0.207*
Compound 6	37.65 ± 0.265*	43.34 ± 0.671*	56.90 ± 0.203*	63.54 ± 0.430*	76.34 ± 0.205*
Compound 7	42.17 ± 0.098*	56.80 ± 0.230*	63.65 ± 0.103*	74.12 ± 0.130*	87.54 ± 0.088*
Compound 8	37.09 ± 0.124*	42.87 ± 0.241*	56.98 ± 0.321*	65.12 ± 0.324*	79.43 ± 0.234*
Compound 9	12.66 ± 0.669*	22.69 ± 0.726*	34.36 ± 0.046*	35.94 ± 0.456*	39.18 ± 0.563*
Compound 10	32.66 ± 0.002*	49.09 ± 0.023*	61.12 ± 0.023*	78.06 ± 0.035*	82.76 ± 0.043*
Compound 11	66.26 ± 0.786*	69.64 ± 0.372*	85.86 ± 0.462*	87.48 ± 0.163*	92.90 ± 0.317*

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

TABLE: 2 *IN –VITRO* FREE RADICAL SCAVENGING EFFECT OF L-ARGININE ANALOGUES BY DPPH SCAVENGING METHOD

Compound	Concentration (µM)				
	% scavenging (Mean ± SEM)				
	25	50	75	100	125
Standard (Ascorbic acid)	78.40 ± 0.246*	81.06 ± 0.208*	85.15 ± 0.205*	89.61 ± 0.192*	93.35 ± 0.198*
Compound 1	23.32 ± 0.231*	28.45 ± 0.241*	34.65 ± 0.210*	44.09 ± 0.165*	54.87 ± 0.187*
Compound 2	20.08 ± 0.320*	24.76 ± 0.231*	37.09 ± 0.287*	48.05 ± 0.230*	53.87 ± 0.812*
Compound 3	1.6 ± 0.209*	4.4 ± 0.061*	11.4 ± 0.186*	20.55 ± 0.096*	35.09 ± 0.225*
Compound 4	35.87 ± 0.243*	47.09 ± 0.280*	58.68 ± 0.023*	68.56 ± 0.076*	78.43 ± 0.021*
Compound 5	21.09 ± 0.021*	28.08 ± 0.023*	32.08 ± 0.210*	44.08 ± 0.021*	56.09 ± 0.023*
Compound 6	28.06 ± 0.231*	32.07 ± 0.023*	43.07 ± 0.023*	58.87 ± 0.213*	65.43 ± 0.210*
Compound 7	37.09 ± 0.023*	45.98 ± 0.213*	57.67 ± 0.287*	65.89 ± 0.276*	78.32 ± 0.234*
Compound 8	39.08 ± 0.243*	48.76 ± 0.265*	59.76 ± 0.254*	73.67 ± 0209*	87.43 ± 0.213*
Compound 9	7.58 ± 0.309*	13.84 ± 0.153*	18.07 ± 0.061*	24.48 ± 0.086*	39.65 ± 0.225*
Compound 10	21.45 ± 0.021*	34.76 ± 0.023*	46.98 ± 0.026*	54.76 ± 0.234*	69.07 ± 0.231*
Compound 11	31.04 ± 0.052*	42.4 ± 0.148*	44.6 ± 0.145*	59.39 ± 0.059*	66.7 ± 0.242*

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

RESULTS AND DISCUSSION: All the L-Arginine analogues synthesized have good yield value. The melting points of all the compounds were determined in an open capillary tube using an electro thermal digital meting point apparatus and are uncorrected. The compounds were characterized using analytical techniques such as UV, TLC and FT-IR. All the Compounds were screened for anti-oxidant activity and the results were compared with that of the standard drug. The study revealed that Compounds4, 5, 6, 7, 8, 10, 11 exhibited very significant anti-oxidant action in NO scavenging method and compounds4, 6, 7, 8, 10, 11 has exhibited significant anti-oxidant activity in DPPH scavenging method. The compounds can be screened for anti-oxidant activity using other screening models to assess their activity on a broader scale which is our future part of the research work.

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