SYNTHESIS, ANTIOXIDANT AND ANTHELMINTIC ACTIVITY OF THE LINEAR TETRAPEPTIDE 
L-(Leu-Pro-Gly)-D-Ala (LPGA)

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ABSTRACT
The objective of the work is to design and synthesize a bioactive tetrapeptide based on Glaucapeptide, a natural heptapeptide. The method includes synthesis of the tetrapeptide L-(Leu-Pro-Gly)-D-Ala (LPGA) by solution phase peptide synthesis using o-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) as a coupling reagent and triethylamine as the base.

The synthesized compound was characterized by FT-IR, 'H-NMR and FAB-Mass and was evaluated for antioxidant and anthelmintic activities. The linear tetrapeptide exhibited good antioxidant activity and potent anthelmintic activity.

Keywords: Tetrapeptide, Solution phase peptide synthesis, Antioxidant activity, Anthelmintic activity.

INTRODUCTION
The linear tetrapeptide L-(Leu-Pro-Gly)-D-Ala (LPGA) is a part of the naturally occurring cyclic heptapeptide, Glaucapeptide A, isolated from the seeds of Annona glauca belonging to the family, Annonaceae. Plants of Annonaceae family are used in folk medicine in various capacities, such as antitumoral, paracitidal, antidiarrhoeal agents, etc. Glaucapeptide A contains L-Alanine in the peptide sequence; however it has been replaced with D-Alanine in the title compounds in order to enhance the biological activity[5-7].

In order to synthesize, the molecule was disconnected into two dipetide units, which were synthesized and coupled using solution phase technique. The method includes the introduction of tertiary butyl oxy carbonyl group (Boc) into amino acids to protect the amino group forming Boc-amino acid. The protection of the carbonyl group was done by converting into corresponding methyl ester. The protected amino acids were coupled using TBTU and triethylamine to get the protected dipeptides. The Boc-group was removed by trifluoroacetic acid and the ester group was removed by lithium hydroxide to get the desired tetrapeptide, LPGA. The structure of the tetrapeptide was confirmed by FT-IR, 'H-NMR and Mass spectra. The synthesized tetrapeptide was evaluated for antioxidant and anthelmintic activities. The compound showed good antioxidant and potent anthelmintic activities.

MATERIALS AND METHODS
Analytical grade solvents and commercially available reagents were used without further purification. Anhydrous conditions for all the reactions were conducted in dried apparatus. All the reactions were magnetically stirred unless otherwise stated. Organic extracts were dried over anhydrous sodium sulphate. Melting points were determined by open capillary method. Amino acids, di-tertbutyloxycarbonyl, trifluoroacetic acid and TBTU were obtained from Spectrochem Ltd., Mumbai. DPPH, Diethyl ether, Methanol and Chloroform was obtained from AVRA. IR spectra were recorded on FTIR spectrometer using a thin film support on KBr pellets. The values are reported as v max (cm^-1). 'H NMR spectra was recorded on 'H NMR Bruker (400MHz) NMR spectrometer. The spectra was obtained in CDCl3 and the chemical shift values are reported as values in ppm relative to TMS (6=0) as internal standard. FAB Mass spectra were recorded. The protection of amino and carbonyl group and their deprotection were done by standard procedures[6-8].

Preparation of Dipetides
Amino acid methyl ester hydrochloride (10 mmol) was dissolved in CHCl3 (20 ml). To this, triethylamine (4 ml) was added and the reaction mixture was stirred for 15 minutes. Boc-amino acid (10 mmol) in CHCl3 (20 ml) and TBTU (10 mmol) were added with stirring. After 1 hour, the reaction mixture was transferred to a separating funnel and was washed with 5% NaHCO3 (20 ml), 5% HCl (20 ml) and distilled H2O (20 ml). The organic layer was dried over anhydrous Na2SO4; filtered and evaporated in a vacuum. The residue was purified by recrystallization from CHCl3[9].

Preparation of Linear Tetrapeptide
The ester group of the dipeptide Boc-L-(Leu-Pro)-OMe was removed and the Boc-group of another dipeptide Boc-L-Gly-D-Ala-OMe was deprotected. Both the deprotected units were coupled and to get the protected linear tetrapeptide which was deprotected at both the ends to get the title compound (Scheme 1).

Antioxidant Activity
The synthesized linear tetrapeptide LPGA was screened for antioxidant activity such as free radical scavenging activity by 1,1-diphenyl-2-picryl-hydrazyl (DPPH)[10-13]. This was measured by following method, wherein the bleaching rate of a stable free radical, DPPH, is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 1 ml of 0.1 M methanolic solution of DPPH was added to 3ml of the synthesized sample LPGA, at different concentrations in methanol (25, 50 and 100 mg/ml). The samples were kept in the dark for 30 min after which the absorbance was measured at 517 nm in a UV spectrophotometer (Jasco V-670 spectrophotometer). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid, which is a good antioxidant, was taken as a standard in this study. The tetrapeptide LPGA showed significant free radical scavenging activity at all different three concentrations studied. The capability to scavenge the DPPH radical was calculated using the following equation:

\[ \text{DPPH Scaevenging Activity (%) } = \left( \frac{A_{0} - A_{t}}{A_{0}} \right) \times 100 \]

Where A0 is the absorbance of the control reaction and At is the absorbance in the presence of sample.

Anthelmintic Activity
Anthelmintic activity study was carried out against earthworms (Eudrilus eugeniae) by Gang's method[14]. Suspension of the sample was prepared by triturating the sample with 15% Tween 80 and distilled water and the mixture was stirred using a magnetic stirrer for 30 minutes. The resulting suspension was used for the activity
The suspension was diluted to contain 100 mg in 20 ml of the test sample. The standard drug, mebendazole, was also prepared with the same concentration in a similar way. Earthworms were placed in three petridishes containing 20 ml of each sample, standard drug and control (20 ml suspension of distilled water and 15% Tween 80) respectively at room temperature. The time required for the paralysis and death of the earthworms were noted. The death time was ascertained by placing the earthworms in warm water at 50°C, which stimulated the movement if the earthworms were alive.

Scheme 1
RESULTS AND DISCUSSION

Physical Data and Spectral Analysis

Yield 72.4%; light brown semi-solid; IR spectrum (v/cm⁻¹): 3433.2 cm⁻¹ (OH stretch), 3287.38 cm⁻¹ (NH stretch), 2935 cm⁻¹ (Alip-CH stretch) 1652 cm⁻¹ (C=O stretch); ¹H NMR spectrum (δ ppm): 10.9-11.1 (1H, s, O-H), 7.9-8.0 (2H, s, NH), 1.03 (6H, d, CH₃), 1.86-1.96 (1H, m, CH), 1.76 (1H, t), 4.1 (2H, d, CH₂), 2.05 (2H, d, NH); The molecular ion peak was obtained at 357 (M+2).

Antioxidant activity

The tetrapeptide LPGA exhibited significant antioxidant activity as compared to the standard, ascorbic acid. With this method it was possible to determine the antiradical power of an antioxidant compound by measuring the decrease in the absorbance of DPPH at 517 nm. A color change from purple to yellow indicated that the absorbance decreased when the DPPH was scavenged by an antioxidant through donation of hydrogen to form stable DPPH molecule. Table 1 illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of prepared sample and standards.

Anthelmintic activity

The synthesized compounds have shown potent anthelmintic activity as compared to the standard drug, mebendazole. Table 2 illustrates a significant anthelmintic activity of sample and standards.

Table 1: Antioxidant activity of LPGA

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Absorbance (Std.)</th>
<th>% of inhibition (Std.)</th>
<th>Absorbance (Sample)</th>
<th>% of inhibition (Sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.0916</td>
<td>35.26</td>
<td>0.0995</td>
<td>29.68</td>
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<tr>
<td>50</td>
<td>0.0602</td>
<td>57.45</td>
<td>0.0701</td>
<td>50.45</td>
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<tr>
<td>100</td>
<td>0.0324</td>
<td>77.10</td>
<td>0.0436</td>
<td>67.51</td>
</tr>
</tbody>
</table>

Table 2: Anthelmintic activity of LPGA

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>Concentration (mg)</th>
<th>Paralyzing Time (Mins. Secs.)</th>
<th>Death Time (Mins. Secs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LPGA</td>
<td>100</td>
<td>54.15</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>Mebendazole (Std.)</td>
<td>100</td>
<td>54.15</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

CONCLUSION

The tetrapeptide could be conveniently prepared by TBTU/ Et: N method in less time (1 hour), with a high yield (72%) and in a pure form as compared to other coupling reagents. The synthesized tetrapeptide was characterized by IR, ¹H NMR and FAB-Mass spectral studies. The compound LPGA exhibited significant antioxidant activity due to the presence of labile hydrogen atoms and potent anthelmintic activity probably due to the presence of D-Alanine.

REFERENCES