Evaluation of *In-vitro* antioxidant potency of smaller chain peptides using DPPH radial scavenging activity, Hydrogen peroxide (H_2O_2) scavenging activity, and Reducing power method (RP)

Anjali Saini[1], Kandasamy Nagarajan[1]*, Garima Kapoor[1], Parul Grover[1], Khushi Shah[1], Hariom Tomar[1]

[1]KIET School of Pharmacy, KIET Group of Institutions, Ghaziabad, Uttar Pradesh, India.

anjalikiet1997@gmail.com, rknagaraaja@gmail.com

Abstract

2,2-Diphenyl-picryl-hydrazyl(DPPH) assay, Hydrogen peroxide scavenging (H_2O_2) assay, and Reducing power method (RP) are in-vitro assays, employed to determine the antioxidant potency of test compound [*Pro-Met, Asp-Cys, Pro-Met-Met,* and *Gly-Asn-Arg-Ala*] using Riboflavin as the standard drug. The percentage of inhibition with different specific concentrations was determined and the IC_{50} value of the test compound was compared with standard, Riboflavin. Among the compound tested in both methods, test drug-4 (*Gly-Asn-Arg-Ala*) has shown maximum potency with an IC_{50} value 3.5µg/ml through DPPH assay and 1.9 µg/ml with reducing power method whereas, in H_2O_2 assay the (Pro-Met-Met) shows maximum potency with IC_{50} value 5 µg/ml and both have shown better antioxidant capacity with that of standard drug Riboflavin (IC_{50} value 1.8 µg/ml and 5.9 µg/ml). Based on the above results, Pro-Met-Met and Gly-Asn-Arg-Ala could be considered for various formulations of antioxidant effect suitable for prevention of bacterial disease for human.

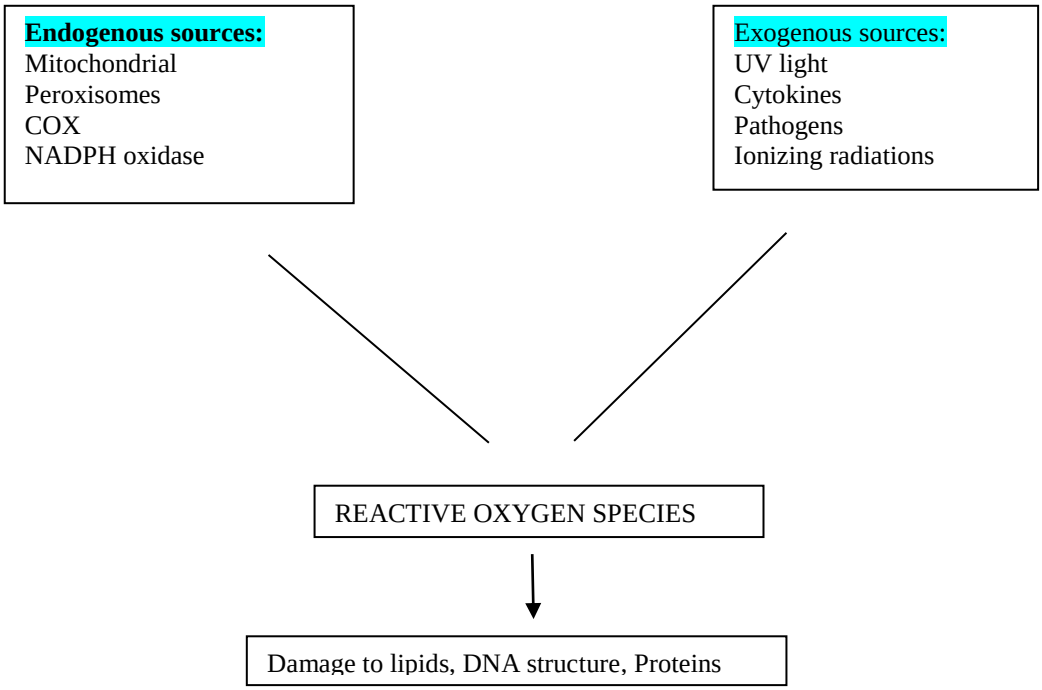
Keywords: *1-Diphenyl-2-picryl-hydrazyl, Hydrogen peroxide, Peptide, Reducing power, Riboflavin.*

*Author for correspondence
Prof. (Dr.) Kandasamy Nagarajan
Address: KIET School of Pharmacy, KIET Group of Institutions, 13 km milestone, Ghaziabad (U.P), 201206, India
Phone: +91-9997628670
E-mail addresses: rknagaraaja@gmail.com, anjalikiet1997@gmail.com

1. Introduction

In this 21st century, various diseases can cause harmful infections in living organisms. There is big attention over oxidative stress various host cells are damaged by the activation of free radicles which is generated from oxidation. Approximately, 250 million people were afflicted on long term basis effects of oxidative stress.

One of the mechanisms referred to here is alterations of mitochondrial membrane, oxidative metabolism, and tricarboxylic acid cycle into cellular membrane showing vulnerability to an invasion of microbes that establish the infection in host cells [1]. Elevated oxidative stress in a host cell damages DNA and protein structure by forming reactive oxygen species (ROS), superoxide radicals, and hydroxyl radicals. They cause extensive damage to cells and lead to increase disease conditions like cancer [2], atherosclerosis [3], inflammation [4], senile dementia, and degenerative eye disease. Host cells have various enzymatic defenses like superoxide dismutase, glutathione reductase, glutathione peroxidase, and repairing system to protect from oxidative stress due to disruption or formation of free radicals whereas these antioxidants are not enough to protect host cells [5].



Oxygen has its benefits and potentially damaging side effects in living organisms. Free radicals are the reactive compounds produced in the human body. Exerts positive effects on our immune system whereas negative effects harm host DNA and proteins. An imbalance between free radicals and antioxidant damage the immune system and leads to increases in oxidative stress or else oxidative damage inflicts through reactive oxygen species known as oxidative stress [6]. This would be affected amino acids which are already present in the host cell for maintaining the immune system [7].

The standard drug which was used in our study riboflavin is an antiviral drug and its mechanism is to defend against viral attacks on host cells but overproduction of riboflavin has genotoxic effects and induces oxidative DNA damage to host cells.

So, we developed the most promising lead known as peptides. A study was designed to investigate the in-vitro antioxidant activity of the 4 peptide test compounds and to establish the most potent antioxidant drug having great therapeutic value.

(Proline-Methionine, Aspartic acid-Cysteine, Proline-Methionine-Methionine, Glycine-Asparagine-Arginine-Alanine) which shows antioxidant property to protect from microbial infections as compare from standard drug Riboflavin.

We were designed a study to explore an anti-oxidant study of selected four smaller chain peptides (Pro-Met, Asp-Cys, Pro-Met-Met, Gly-Asn-Arg-Ala) as test compounds and prove that the most potent smaller chain peptides assess the significant antioxidant property and maintain the immune

system in host cells with reducing inflammation and allergies along with marked protection from microbes.

2. Materials

All the chemicals like DPPH, Riboflavin as a standard drug was procured from CDH Lab., New Delhi. Ethanol solution was used to form purple color solution of DPPH for measuring the radial scavenging activity. Whereas, an analytical grade of chemical like hydrogen peroxide (light sensitive drug) were used to measure scavenging activity of all peptides.

3. Methods

2,2-diphenyl-1-1-picrylhydrazyl (DPPH) radical scavenging activity is an in-vitro antioxidant activity shows/measure the inhibition concentration at 50% of population are as follows:

a) Bleaching of the purple colored ethanol solution of DPPH (Ignatowicz et al., 1994) [8] 0.1mM solution of DPPH in ethanol was prepared.
b) Then, 0.1ml of DPPH solution was added to 3ml of different concentration(1 μg/ml, 2 μg/ml, 4 μg/ml, 8 μg/ml, 16 μg/ml, and 32 μg/ml) of the test drug -1,2,3,4.
c) After 30 minute incubation period at room temperature, the absorbance was read against a blank at 517nm using UV -visible double beam spectrophotometer (Shimadzu-1800) through software UV probe.
d) Percentage of scavenging activity of different concentration of test drug was determined
e) IC_{50} value of test drug was compared to that of standard drug Riboflavin.

The IC_{50} value defined as the concentration of test drug that inhibits the formation of DPPH radicals by 50% (Manzocco et al., 1998)[9]**.**

Next method was **Hydrogen peroxide scavenging (H2O2) assay** [10], this help to evaluate or measure the scavenging activity and procedure were follows:

1) Solution of hydrogen peroxide (40mM) is prepared in phosphate buffer (50mM pH 7.4).
2) Different concentration of test drug in distilled water was added to hydrogen peroxide and absorbance was determined at 230nm after the 10 minutes against the blank solution containing phosphate buffer without hydrogen peroxide using Ultra Voilet visible double beam spectrophotometer.
3) Then determined the % of scavenging activity at different concentrations of test drug was compared with that of Riboflavin, which was used as a standard.

Another last method was **reducing power method assay** [11], this help to evaluate or measure the scavenging activity and procedure were follows:

1) A colored complex with potassium ferricyanide, trichloroacetic acid and ferric chloride- forms antioxidant compound.
2) Then 2.5mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v) was added to 1.0 mL of sample dissolved in distilled water.
3) Resulting mixture was incubated at 50 degree Celsius for 20 minutes, which was followed by addition 2.5mL trichloroacetic acid (10% w/v).
4) The mixture was centrifuged at 3000 rpm for 10 minutes to collect the upper layer of the solution (2.5mL)
5) The mixed with distilled water and add 2.5mL ferric chloride (0.1% w/v).
6) The absorbance was measured at 700nm using UV spectrum (shimadzu -1800).
7) The % of scavenging activity was measured and compared with standard drug riboflavin.

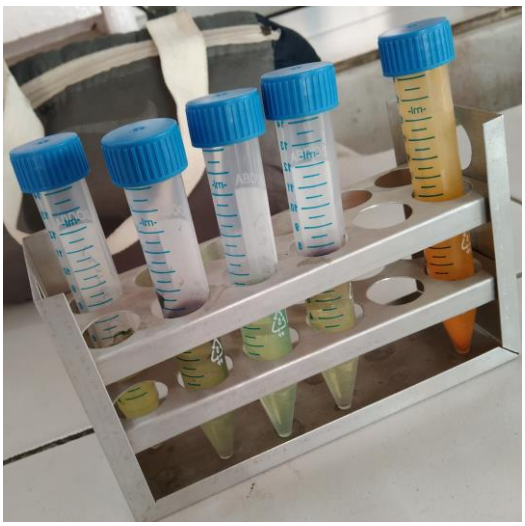


Image of centrifuge tubes with test compounds and standard drug riboflavin.

Refrigerated centrifuge

4. Results and Discussion:

The results of DPPH radial scavenging activity of the subjected peptide leads are shown in table 1.

Table 1. DPPH radial scavenging activity for Peptides

S.No.	Compound Name	% Inhibition (µg/ml)						IC_{50} (µg/ml)
		1	2	4	8	16	32	
1	Test compound (Pro-Met)	32.0	27.2	16.6	13.2	10.2	100	13
2	Test compound (Asp-Cys)	38.5	24.1	11.7	10.8	10.2	100	15.4
3	Test compound (Pro-Met-Met)	35.6	25.2	11.7	10.3	10.4	100	10
4	Test compound (Gly-Asn-Arg-A	32	27	16.6	13.2	10.2	100	3.5
5	Standard (Riboflavin)	33	53	97	100	94	96	1.8

value obtained from regression lines with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. All values given are mean of triplicate experiment at S.D (5%) for the above table.

All the test drug above 1-4 demonstrated above the DPPH radial scavenging activity was detected in test drug-4 (*Gly-Asn-Arg-Ala*) with an IC_{50} value of 3.5 µg/ml (Table-1), which shows the antioxidant activity greater than that of standard Riboflavin IC_{50} value of 1.8 µg/ml.

Similarly test drug-1 (*Pro-Met*), test drug-2 (*Asp-Cys*), test drug-3 (*Pro-Met-Met*) have shown almost comparatively better antioxidant capacity with that of standard drug Riboflavin with IC_{50} value are 13 µg/ml, 15.4 µg/ml, and 10 µg/ml.

The UV- spectrometric graph was determined of test drug and standard drug with their linear regression and correlations coefficient shown in figure 1-5

Figure 1. Graphical representation of linearity for the standard drug concentration with their corresponding values

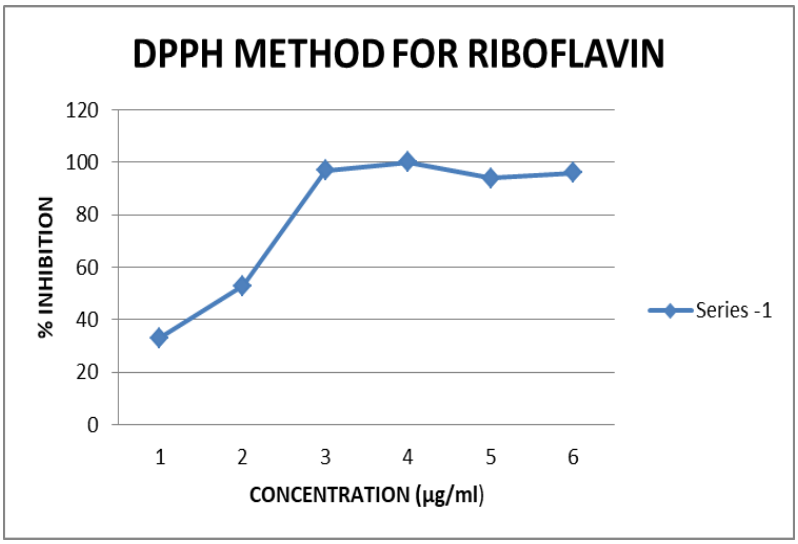


Figure 2. Graphical representation of the test drug 1 (Pro-Met) concentration with their corresponding values

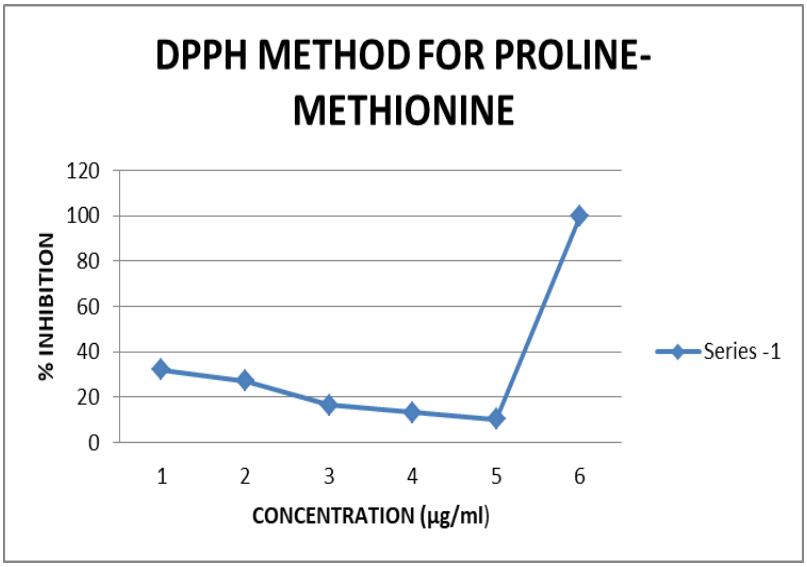


Figure 3. Graphical representation of the test drug 2 (Asp-Cys) concentration with their corresponding values

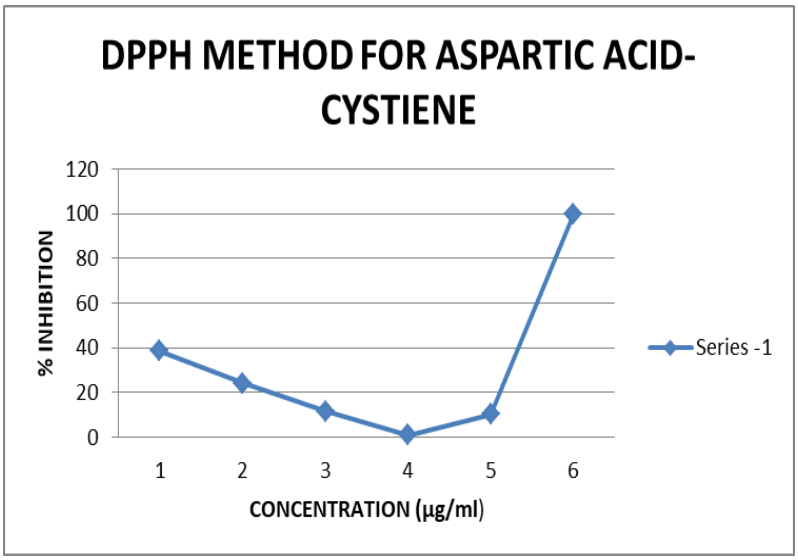


Figure 4. Graphical representation of the test drug 3 (Pro-Met-Met) concentration with their corresponding values

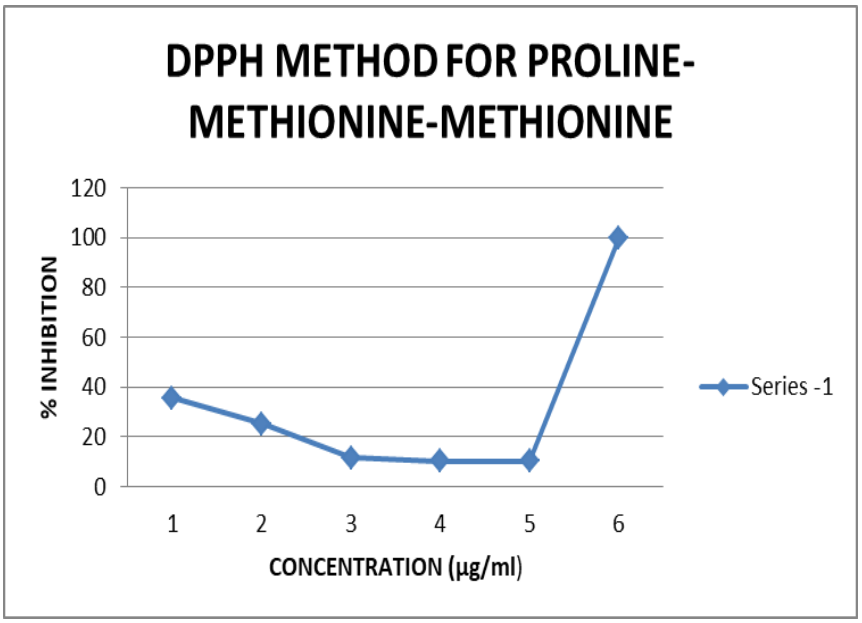
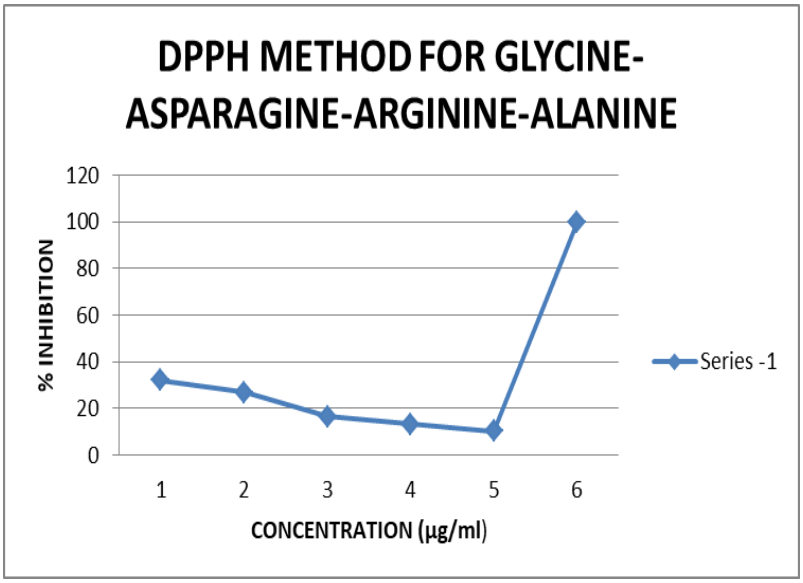


Figure 5. Graphical representation of the test drug 4 (Gly-Asn-Arg-Ala) concentration with their corresponding values



The results of Hydrogen Peroxide scavenging assay of the subjected peptide leads are shown in table 2.

Table 2. Hydrogen Peroxide Scavenging activity for Peptides

S.No.	Compound Name	% Inhibition (µg/ml)						
		1	2	4	8	16	32	IC_{50}(µg/ml)
1	Test compound (Pro-Met)	22.1	20.3	15.1	10.4	10.1	100	6
2	Test compound (Asp-Cys)	14.8	11.7	15.0	10.3	10.2	100	7.4
3	Test compound (Pro-Met-Met)	31.6	27.9	15.9	10.7	10.3	100	5
4	Test compound (Gly-Asn-Arg-Al	62.0	12.1	14.8	11.8	10.3	100	6.5
5	Standard (Riboflavin)	67	24.7	18.5	11.5	12.3	100	5.9

value obtained from regression lines with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. All values given are mean of triplicate experiment at S.D (5%) for the above table.

All the test drug above 1-4 demonstrated above the Hydrogen Peroxide scavenging activity was detected in test drug-3 (*Pro-Met-Met*) with an IC_{50} value of 5 µg/ml (Table-2), which shows the antioxidant activity greater than that of standard Riboflavin IC_{50} value of 5.9 µg/ml. Similarly test drug-1 (*Pro-Met*), test drug-2 (*Asp-Cys*), test drug-4 (*Gly-Asn-Arg-Ala*) have shown almost comparatively better antioxidant capacity with that of standard drug Riboflavin with IC_{50} value are 6 µg/ml, 7.4 µg/ml, and 6.5 µg/ml.

The UV- spectrometric graph was determined of test drug and standard drug with their linear regression and correlations coefficient shown in figure 6-10.

Figure 6. Graphical representation of linearity for the standard drug concentration with their corresponding values

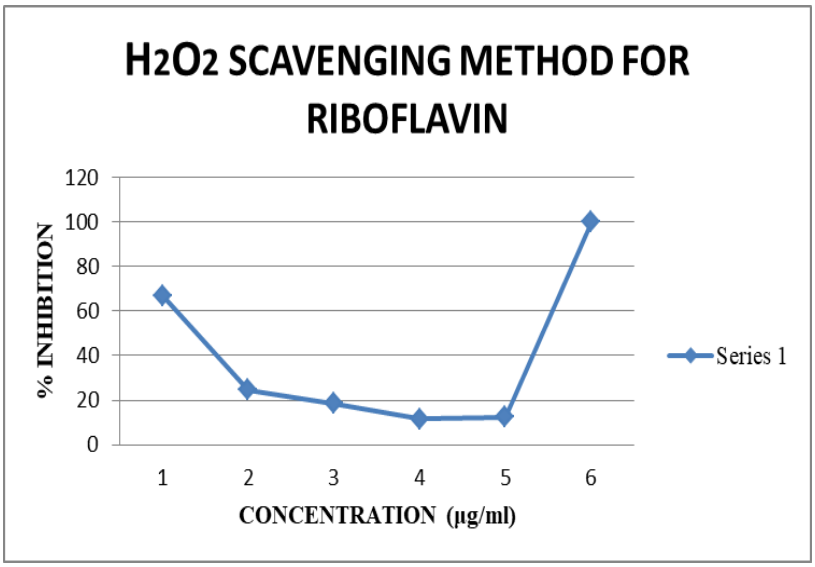


Figure 7. Graphical representation of the test drug 1 (Pro-Met) concentration with their corresponding values

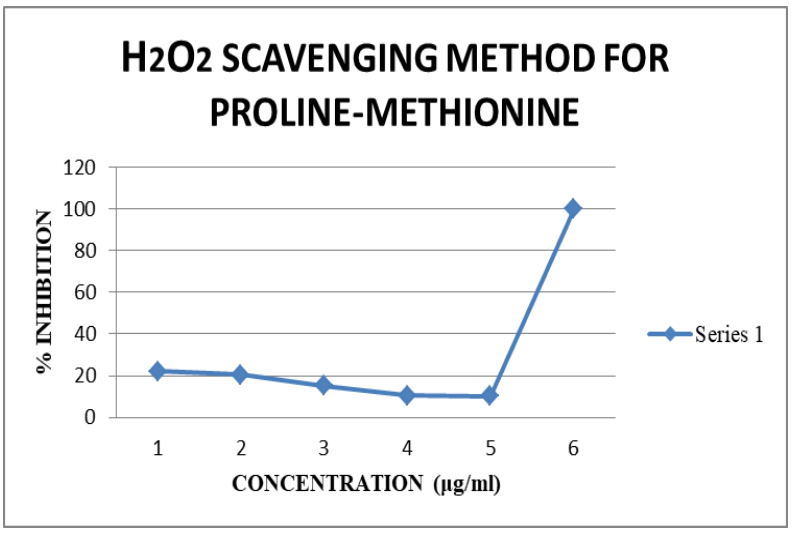


Figure 8. Graphical representation of the test drug 2 (Asp-Cys) concentration with their corresponding values

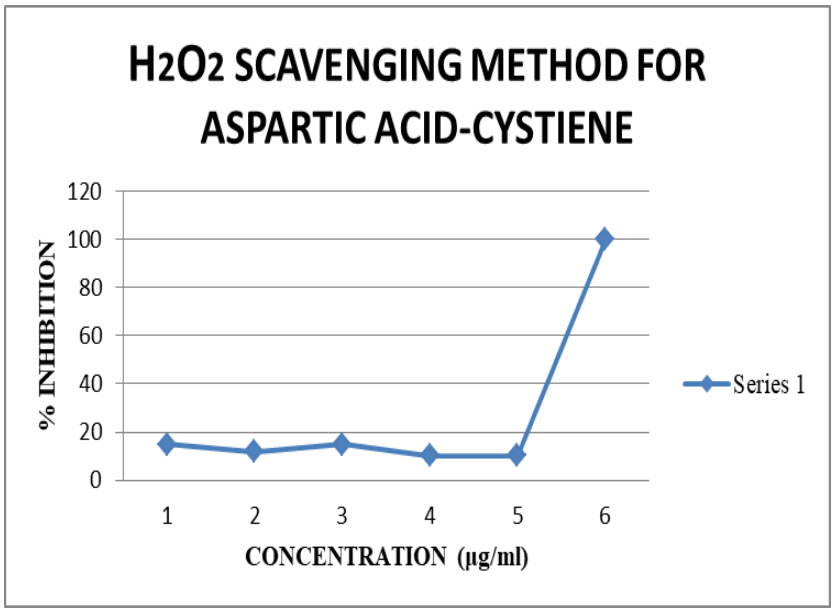


Figure 9. Graphical representation of the test drug 3 (Pro-Met-Met) concentration with their corresponding values

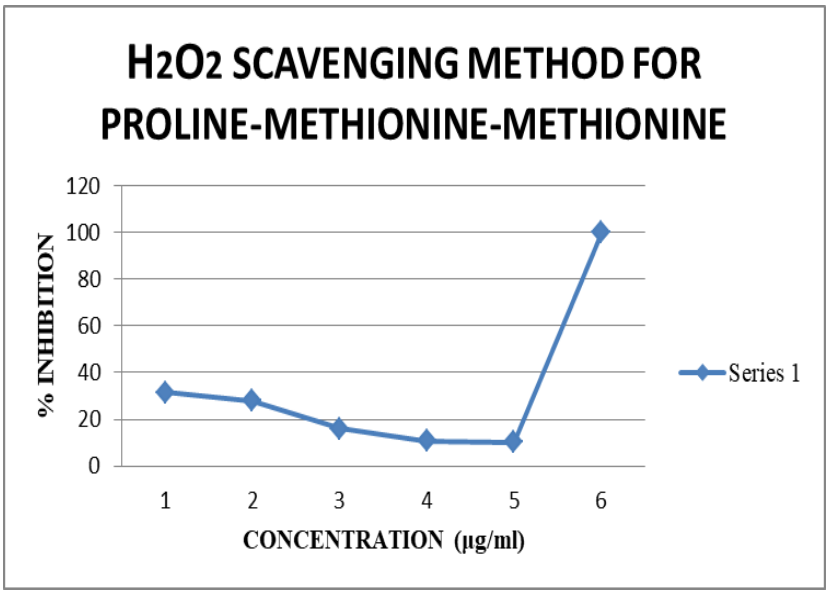
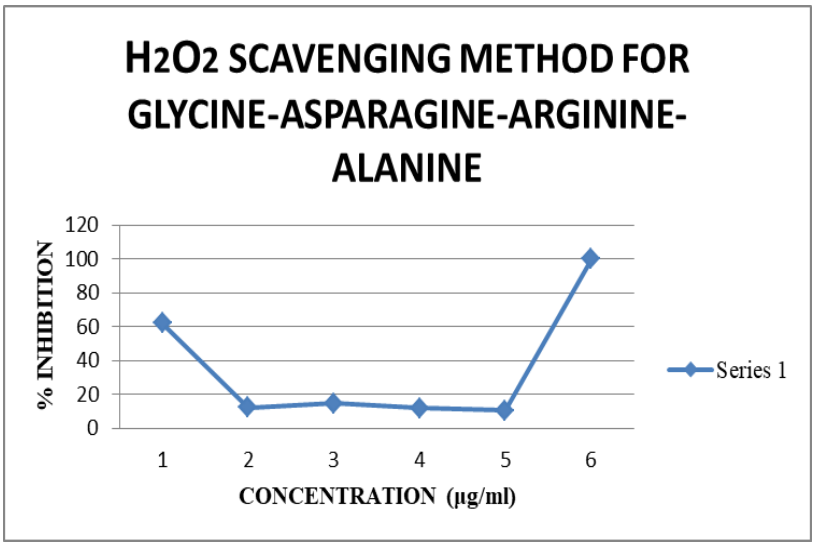


Figure 10. Graphical representation of the test drug 4 (Gly-Asn-Arg-Ala) concentration with their corresponding values



The results of reducing power method assay of the subjected peptide leads are shown in table 3.

Table 3. Reducing Power Method activity for Peptides

S.No.	Compound Name	% Inhibition (µg/ml)						IC_{50}(µg/ml)
		1	2	4	8	16	32	
1	Test compound (Pro-Met)	28.84	21.16	25.22	16.31	12.14	100	15.3
2	Test compound (Asp-Cys)	21.20	22.69	18.86	19.76	11.68	100	14
3	Test compound (Pro-Met-Met)	37.87	43.30	29.01	18.41	11.91	100	7.9
4	Test compound (Gly-Asn-Arg-A	44.63	59.09	17.92	13.27	10.38	100	1.9
5	Standard (Riboflavin)	30.53	10.90	15.74	13.49	12.93	100	14.2

value obtained from regression lines with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. All values given are mean of triplicate experiment at S.D (5%) for the above table.

The entire test drug above 1-4 demonstrated above the Reducing power method activity was detected in test drug-4 (*Gly-Asn-Arg-Ala*) with an IC_{50} value of 1.9 µg/ml (Table-3), which shows the antioxidant activity greater than that of standard Riboflavin IC_{50} value of 14.2 µg/ml.

Similarly test drug-1 (*Pro-Met*), test drug-2 (*Asp-Cys*), test drug-3 (*Pro-Met-Met*) have shown almost comparatively better antioxidant capacity with that of standard drug Riboflavin with IC_{50} value are 15.3 µg/ml, 14 µg/ml, and 7.9 µg/ml [12].

The UV- spectrometric graph was determined of test drug and standard drug with their linear regression and correlations coefficient shown in figure 11-15.

Figure 11. Graphical representation of linearity for the standard drug concentration with their corresponding values

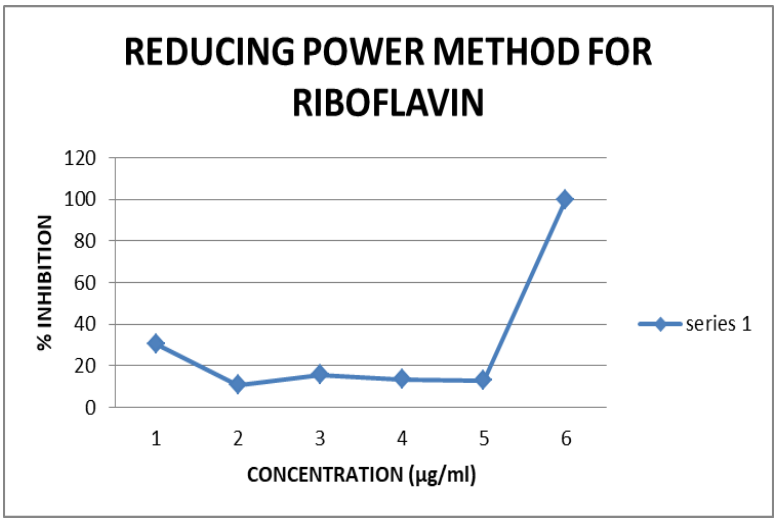


Figure 12 . Graphical representation of the test drug 1 (Pro-Met) concentration with their corresponding values

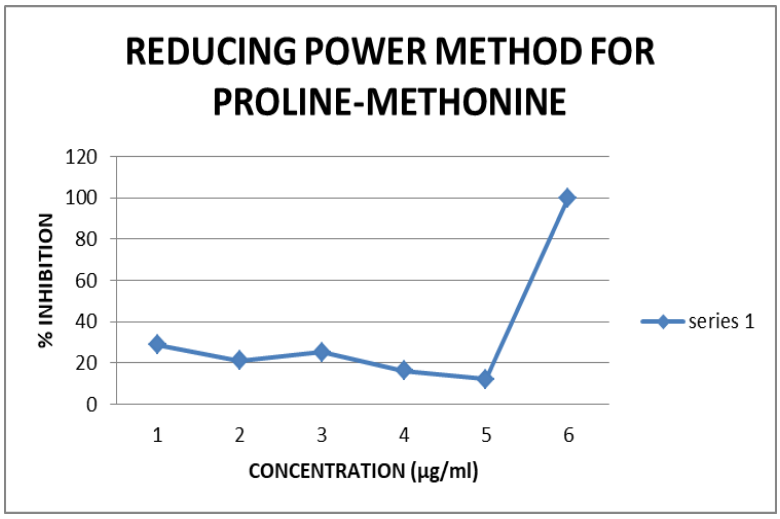


Figure 13. Graphical representation of the test drug 2 (Asp-Cys) concentration with their corresponding values

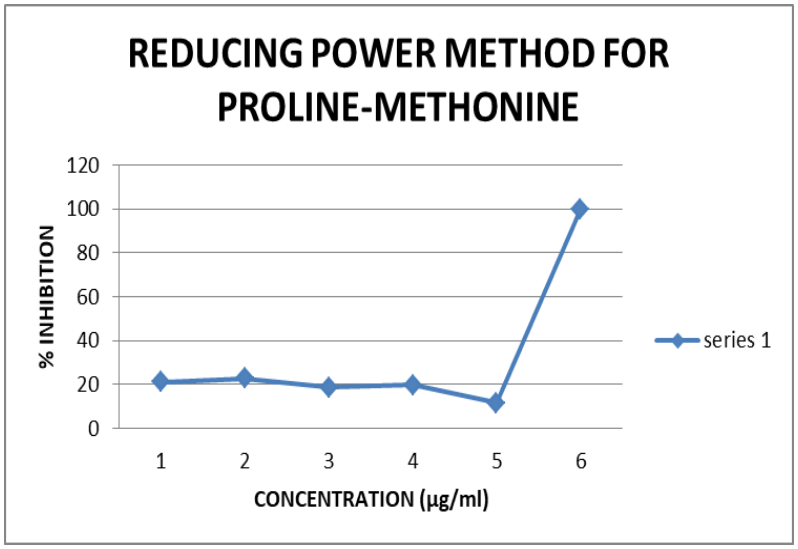


Figure 14. Graphical representation of the test drug 3 (Pro-Met-Met) concentration with their corresponding values

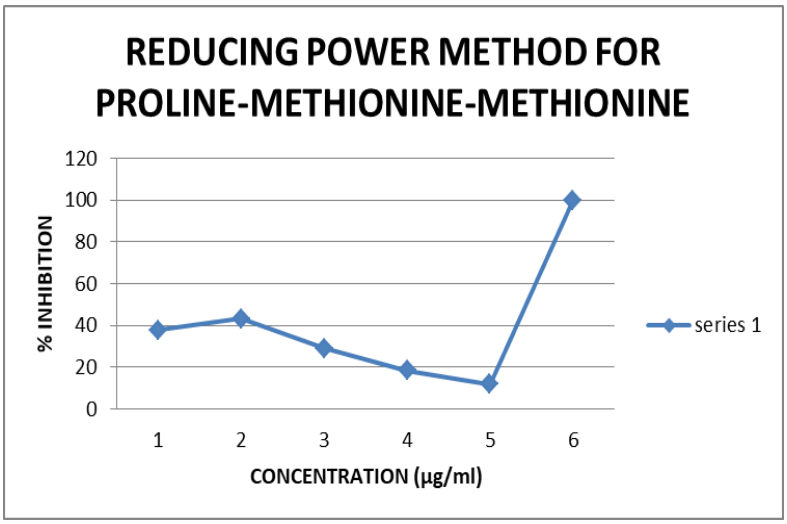
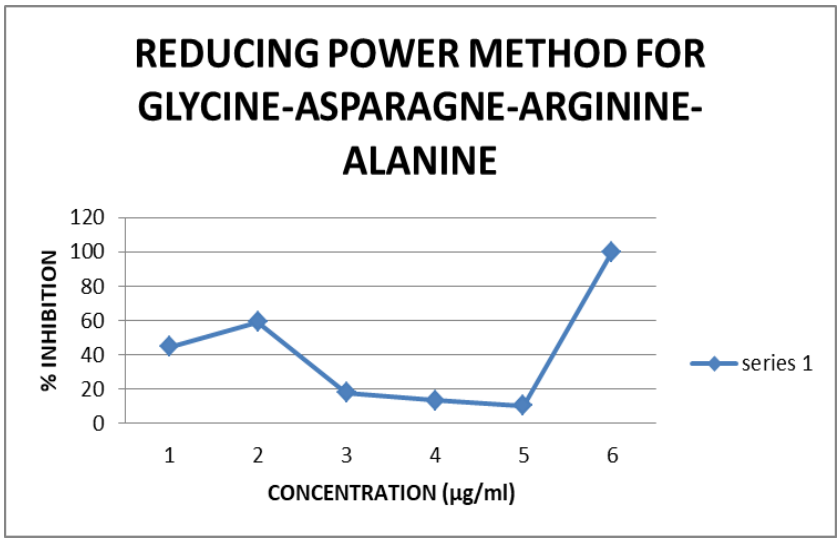


Figure 15. Graphical representation of the test drug 4 (Gly-Asn-Arg-Ala) concentration with their corresponding values



Acknowledgments

The authors acknowledge and are grateful to Dr. (Col.) A. Garg, Director, and Dr. Manoj Goel who is the Joint Director of KIET Group of Institutions in overall support for carrying out the work.

References

[1] *Wang Y, Li N, Zhang X, Horng T. Mitochondrial metabolism regulates macrophage biology. J Biol Chem. 2021 Jul;297(1).*

[2] *Kinnule V.L., Crapo J.D. Superoxide dismutase in malignant cells and human tumors. Free radic.Bio.med. 39:120, 2004.*

[3] *Singh U., Jialal I. Oxidative stress and atherosclerosis. Pathophysiological 13:129-142, 2006.*

[4] *Mao H, Zhao Y, Li H, Lei L. Ferroptosis as an emerging target in inflammatory diseases. Prog Biophys Mol Biol. 2020 Sep;155.*

[5] *Nagarajan, K., Mazumdar,A., Ghosh L.K. In-vitro antioxidant activity of alcholic extraxts of Wrightia tomentosa. Pharmacologyonline, 1:196-203, 2008.*

[6] *Tonoyan L, Montagner D, Friel R, O'Flaherty V. Antimicrobials offered from nature: Peroxidase-catalyzed systems and their mimics. Biochem Pharmacol. 2020 Dec;182:114281.*

[7] *Nuti R, Goud NS, Saraswati AP, Alvala R, Alvala M. Antimicrobial Peptides: A Promising Therapeutic Strategy in Tackling Antimicrobial Resistance. Curr Med Chem. 2017;24(38):4303-4314.*

[8] *Ignatowicz E, Rybczyńska M. Some biochemical and pharmacological aspects of free radical-mediated tissue damage. Polish Journal of Pharmacology. 1994 May-Jun;46(3):103-114.*

[9] *Manzocco L., Anese M., Nicoli M.C. Antioxidant properties of tea extracts as affected by processing. Lebens-mittel-Wissen-schaft Und-Technologie 31 (7-8): 694-698, 1998.*

[10] *Sharma A, Ahmad J, Flora SJS. Application of advanced oxidation processes and toxicity assessment of transformation products. Environ Res. 2018 Nov;167:223-233.*

[11] *Munteanu IG, Apetrei C. Analytical Methods Used in Determining Antioxidant Activity: A Review. Int J Mol Sci. 2021 Mar 25;22(7):3380.*

[12] *Nagarajan, K., Saini, A., Goyal, T.,Grover, P.,Kapoor, G.,Ghai, R., Goel, R., Kumar, V., Sexana, V., Chauhan, D. Computer-aided Drug Design of New Tripeptides as Inhibitors of Mycobacterium Tuberculosis Protein Tyrosine Phosphatase. YMER, 2022 Nov;21: 0044-0477.*