Research article

Synthesis and characterization of 2-thiophen flavonoid analogue for free radical scavenging antioxidant analysis

Prabhulingayya Bhixavatimath[1], Akram Naikawadi[2], Yasmeen Maniyar[1], Gurudatta Moharir[2], Vijayakumar Daroj[3]

[1]Department of Pharmacology, S. Nijalingappa Medical College & HSK Hospital and Research Centre, Bagalkot, 587102, Karnataka, India
[2]Department of Pharmacology, Shri. B. M. Patil Medical College, BLDE (Deemed to be) University, Vijayapura, 586103, Karnataka, India
[3]Department of Pharmaceutical Chemistry, Cauvery College of Pharmacy, Mysore, 570028, Karnataka, India

(Received: January 2022 Revised: May 2022 Accepted: June 2022)

Corresponding author: **Prabhulingayya S. Bhixavatimath**. Email**:** drprabhusb@gmail.com

ABSTRACT

Introduction and Aim: Currently research is focussed on the use of antioxidants in preventing oxidative stress induced diseases. Flavonoids present in plant sources gaining more therapeutic importance due to their antioxidant property, but their solubility and some pharmacokinetic concern, diverted the current research study towards the synthesis of these flavonoids for their therapeutic potential. The study was aimed to synthesize and characterize the 2-thiophen flavonoid analogue for free radical scavenging antioxidant activity.

Materials and Methods: The test synthetic compound PNF(3-hydroxy-2-(thiophen-2-yl)-4H-chromen-4-one) a thiophen substituted flavonoid was synthesized from condensation fallowed cyclization reaction in laboratory and DPPH, superoxide, nitric oxide, and hydroxyl radical scavenging activity was determined through established *in vitro* methods.

Results: It suggests that the test flavonoid (PNF) possesses the potent free radical scavenging on DPPH, superoxide, nitric oxide, and hydroxyl radicals with IC_{50} values of 6.89±25µg/ml,4.04µg/ml, 2.44µg/ml and 2.96µg/ml respectively. The radical scavenging potential of test PNF synthetic compound at different concentrations(10µg-150µg) was compared with that of standard antioxidants such as BHA, ascorbic acid used in the study.

Conclusion: Results from this study indicates that the novel flavonoid PNF exhibited the considerable dose dependant *invitro* antioxidant activity. These possible activities could be useful to consider the novel synthetic thiophen derived flavonoid as therapeutic antioxidant agent.

Keywords: Antioxidant; DPPH; flavonoid; hydroxyl; nitric oxide.

INTRODUCTION

In recent years, there is an increase attention towards antioxidants usages in oxidative stress induced diseases, because of their protection over various cells, their organelles and even over metabolic pathways from the harmful oxidative radicals (1, 2). Free radicals are electrically charged unpaired electrons. These radicals being highly unstable, become neutralized when they pick up the electrons from other surrounding biological substances. During this neutralization reactions though earlier radical was deactivated, subsequently newer radical will be produced, leading cascade of radical reaction. Within a fraction of time several free radicals can be generated. Reactive oxygen species (ROS) such as O_2,H_2O_2, peroxyl radicals, OH^- and nitrogen species are most common free radicals usually synthesized during normal cellular metabolism (3). At physiological levels these free radicals play a key role in controlling cell viability, cell signaling, cellular differentiation, protecting cells through killing and degradation of pathogenic organisms (4). But if these free radial levels go beyond normal levels and in presence of weak antioxidant system, leads to the development of oxidative-antioxidant imbalance and oxidative stress induced diseases. Though the body has its own mechanism to combat oxidative stress, when exposure is more than body's antioxidant capacity, the problem aggravates (5) and therefore exogenous antioxidants which alleviate the harmful effects of free radicals are important (6).Antioxidants are a group of compounds that neutralize or prevent free radicals or reactive species and avoid cell or tissue damage caused by oxidative potential of free radicals (7). Although several indigenous molecules have been proved for their antioxidant activities (8) only few of them have been used clinically. Hence, there is a demand to explore new molecules with antioxidant properties to combat oxidative stress.

Flavonoids are polyphenolic compounds present in herbal food products, are considered as natural antioxidants as they are one of the important constituent present in animal and human diet (9,10).

Though, natural flavonoids being potent antioxidants but have limitation in terms of standardization procedure, instability, solubility concern pharmacokinetic properties (11). Hence a novel test flavonoid was synthesized in laboratory to determine its scavenging antioxidant potential against hydroxyl, nitric oxide, and superoxide anion free radicals in *invitro* methods.

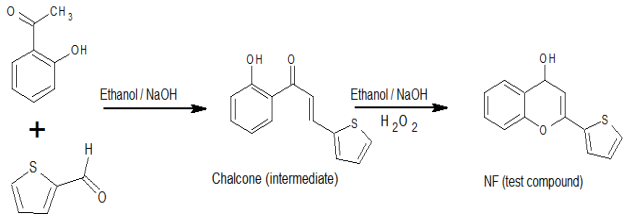
MATERIALS AND METHODS

2-Hydroxyacetophenone and 2-thiophen benzaldehyde(Sigma Aldrich), 1,1-diphenyl-2-picrylhydrazyl, methanol, disodium hydrogen phosphate(Na2HPO4), NADH, EDTA, butylated hydroxy anisole(BHA), nitrobluetetrazolium (NBT), TBA, 2-deoxy-2-ribose, trichloroacetic acid, phenazine methosulphate, potassium ferricyanide and standard ascorbic acid, were obtained from SD fine chemicals and Sigma Loba chemicals.

Synthesis of test flavonoid compound (PNF)

The compound was synthesised by usingAlgar-F-Oyamada method(12)and spectralcharacterization for IR, NMR and Mass spectroscopy was done after recrystalization procedure.

Chemical reaction and proposed structure of test PNF compound drawn from chemsketch software



PNF-3-hydroxy-2-(thiophen-2-yl)-4H-chromen-4-one

Analytical methods

Extent of the reaction and purities of the obtained products were assessed by using TLC plates (Merck 60 F254). The test compound was analyzed for elemental analysis. Open capillaries method was used to determine the melting point in^{0}C and uncorrected. KBr disc method was employed to get IR spectra through FTIR spectrometer-8300(Shimadzu, Japan) and FTIR 4100.^{1}H-NMR and^{13}C-NMR spectra were recorded on High resolution Ft-MR Multinuclear SpectrometerBrukar) in deuterated chloroform with internal standard tetramethylsilane(TMS), working at 400MHz and 75MHz frequencies, respectively.

Chemical shift readings are presented in δ (ppm)downfield, with respect to an internal standard TMS. GC-MS-QP5050A (Shimadzu) was used to assess the Mass spectral data of the test compound (PNF) in quality assurance department, at Manipal College of Pharmaceutical Sciences Manipal. Structural and analytical data are presented in Table 1. Spectral data of the synthesised compound is given in Table 2.

In vitro free radical scavenging activity

Various *in vitro* procedures were employed to determine the free radical scavenging property of novel synthetic flavonoid (PNF) as given below,

DPPH radical scavenging activity(13)

The free radical scavenging capacity of the test drug PNF and standard drug Butylated Hydroxy Anisole (BHA) was determined using stable DPPH radical method.

Methanolicsolutions of flavonoid and Butylated Hydroxy Anisole (BHA) in dimethyl sulfoxide (DMSO4) at various concentrations (10µg/ml, 50µg/ml, 100µg/ml and 150µg/ml) were added to 0.5ml of 0.1 mM methanolic solution of 0.004% DPPHin test tubes separately and were allowed to stand at room temperature for 20 min. A 0.1 mM methanolic solution of DPPH without test was used as control, whereas Butylated Hydroxy Anisole was employed as reference standard. After observing de-colorization of DPPH, the absorbance's of samples were determined at 517 nm. The percentage radical scavenging activity was calculated by using the formula:

DPPH scavenged % = $(A_{Cont} - A_{Sample})/A_{Cont}$ X100; A_{Cont} and A_{Sample} are the absorbance values at 517 nm. Lower absorbance values suggest high radical scavenging action.

Hydroxyl(OH^*) radical scavenging assay

This was performed according to a method reported by Klein *et al.*, (14**).** To a glass tube containing 1 ml of various concentrations (10µg/ml, 50µg/ml, 100µg/ml and 150µg/ml)of test flavonoid (PNF) & Ascorbic acid (AA), 1ml of iron-EDTA solution(0.13% ferrous ammonium sulphate 0.26% EDTA), 0.5ml of EDTA solution, 1ml of DMSO (0.85% in 0.1 mol/L phosphate buffer pH 7.4),0.5ml of 0.22% ascorbic acid was added to start the reaction and this was caped tightly & heated in a boiling water bath at 80°-90°C for 15 min. After this ice-cold TCA (17.5%) was added to stop the reaction. Then 3 ml Nash reagent solution was added to the above reaction mixture, and incubated at room temperature for 15 min for colour change.

By using spectrophotometer, the intensity of the yellow colour absorbance was measured at 412 nm against a reagent blank. As reference standard Ascorbic acid (AA) was used. The % hydroxyl radical scavenging activity (%HRSA) was calculated using the formula:

$$\% \text{ HRSA} = [(A_{control} - A_{sample}) / A_{control}] \text{ X } 100.$$

Nitric oxide (NO^*) scavenging assay

The colorimetric Griess reaction method described by Sun J*et al* was used to determine the test flavonoid and standard reference drug's ability to inhibit NO radical generated from sodium nitroprusside (15). The sodium nitroprusside (10 mM, 4 ml)) in phosphate buffer saline (7.4 pH 1ml), was mixed with 1 ml of test compound and standard BHA drugs dissolved in methanol at different concentrations (10µg/ml,50µg/ml, 100µg/ml and 150µg/ml) were incubated at 25ºC for 150 min. After this, 1.5 ml of incubated solution containing nitrate was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) and allowed to stand for 30 min in dark place. The absorbance of the chromophore formed during the diazotization of the nitrite with sulphalinamide and the subsequent coupling with napthyethylene diamine dihydrochloride was measured at 546nm. All the tests were performed in triplicate. Percentage NO inhibition was calculated by using the following formula:

% NO radical scavenging activity= (control OD – sample OD) ×100 /control OD

Superoxide anion radical scavenging activity

Superoxide radical inhibition activity was performed using the method described by Jia *et al.*,(16) with slight modification. In this assay, the measurement of nitroblue tetrazolium(NBT)through spectrophotometer is important. The test synthetic flavonoid (PNF) and reference standard ascorbic acid (AA) in phosphate buffer (0.05M and pH 7.8) at different concentrations (10µg/ml, 50µg/ml, 100µg/ml and 150µg/ml) were added to the respective test tubes containing mixture of 1ml of nitroblue tetrazolium(150 µM)and 1ml of nicotinamide adenine dinucleotide(234 µM)in Tris-HCl buffer 16mM pH 8.0; Then to start the reaction, 1ml of 40 µM PMS (phenazine methosulphate) solution was added to each of the above test tubes containing reaction mixtures and incubated at room temperature for 5 min. The samples optical density was measured at 560 nm. Percentage super oxide radical scavenging activity was calculated by using the following formula: % SO radical scavenging activity = (control OD - sample OD) ×100/ Control OD.

Statistical analysis

One-way analysis of variance(ANOVA), followed by multiple Dunnet comparison tests were used for statistical analysis of the results. $p<0.001$ was considered significant. Data are represented as mean ± S.E.M (n=3).The IC_{50} values for test compound as well as reference standards were calculated using the Microsoft excel.

RESULTS

In the present work, Claisen – Schmidt's condensation reaction between 2 hydroxy acetophenone and thiophen benzaldehyde yielded an intermediate chalcone. Then cyclisation reaction of this chalcone was made with hydrogen peroxide in alkaline medium to get the final product of our interest that is flavone. The progress of reaction and purity of test compound was monitored on TLC plates in appropriate solvent (20% Ethyl acetate in n hexane). The molecular formula, chemical name and experimental data of synthesized test compound were reported in Table1.The purified test compound PNF, characterized by IR, NMR and mass spectroscopy. The spectral data PNF test compound supported the structures and properties of proposed flavonoid compound.

DPPH free radical scavenging activity

It is one of the standard assay among the radical scavenging activity studies as it provides rapid results for the radical inhibition activity of particular compound(17). The results of DPPH scavenging activity of test (PNF) and standard BHA drug are depicted in Fig**.**1.In this study, the novel synthetic flavonoid (PNF) exhibited the significant radical scavenging activity in a dose dependent manner and it was comparable to standard BHA. At 150µg dose the synthetic flavonoid (PNF) and standard BHA showed about 34.12 % and 93.21 % of radical inhibition activity respectively.

Table 1: Substitution pattern moieties on ring A and B structure of the flavonoid compound (PNF)

Code	R-H	Elemental analysis	Molecular formula	IUPAC Name	MW	% yield	Melting point(^{0}C)	Rf* Value
PNF	R^1-H R^2-H R^3-H R^4-H	C (63.92%) H(3.30%) O (19.65%) S(13.13%)	$C_{13}H_8O_3S$	3-hydroxy- 2-thiophen-2-yl-4H-chromen-4-one	244	67	196-199	0.63

Table 2: Spectral data of synthesized compound PNF

Code	IR (KBr)ν(cm-1)	^{1}H NMR(CDCl3)/ δ in ppm	^{13}C NMR(CDCl3) / δ in ppm	Mass spectra
PNF	3227,(b,OH), 1610(C=O), 1560,1479,1346, 1294,1120–(H of Ar)	12.37 (1Hs OH) and 6.98 – 8.10 (mH of ArH)	193.17(Cs,C=O);and 129-140,(Cm, of Ar).	244.26 Da

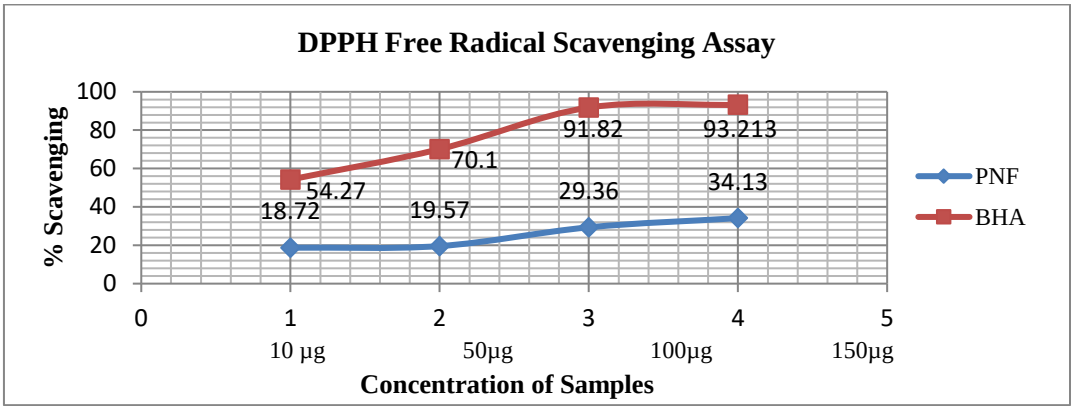


Fig. 1. DPPH free radical percentage scavenging activity of test drugs such as PNF and BHA at different concentrations

The IC_{50} of standard BHA and test synthetic flavonoid (PNF) is around 53±01µg and 6.89±25µg respectively which suggest that the synthetic flavonoid test drug is less potent scavenging antioxidant than the standard drug.

NO radical scavenging activity

The test PNF compound's NO scavenging activity results were summarized in Fig. 2. From the analysis, the synthetic novel flavonoid (PNF) showed the highest (46.08%) radical inhibitory effect at 150µg /mL with the IC_{50}value of 4.04 µg /ml. Whereas, the standard antioxidant BHA at150µg /ml concentration, showed the highest NO radical scavenging activity up to 54.34 % with the IC_{50} of 3.41 µg/ml Fig. 2 explains about the NO radical inhibitory activity of test flavonoid. The test flavonoid (PNF) showed a low to moderate nitric oxide scavenging activity. As the concentration of synthetic flavonoid (PNF) increases, it's percentage radical scavenging activity also increases.Therefore, NO radical scavenging activity of synthetic novel flavonoid (PNF) was quite comparable to BHA at a concentration of 150µg/mL.

Hydroxyl radical scavenging activity

Among the reactive oxygen species, hydroxyl radical is most common, causing severe damage to adjacent biomolecule. By generating OH^- radicals using Ascorbic acid–iron EDTA reaction mixture, hydroxyl radical scavenging activity was determined. Fig.3. shows the hydroxyl radical scavenging activity of various concentration of test PNF and standard ascorbic acid. The PNF at 150µg concentration exhibited highest hydroxyl radical scavenging activity which is comparable to standard ascorbic acid. The IC_{50} values of test synthetic novel flavonoid (PNF) and Ascorbic acid (AA) were 2.96 and 0.88 µg/mL respectively. The test synthetic novel flavonoid (PNF) showed the increasing order of OH^- scavenging potential in a dose dependent manner.

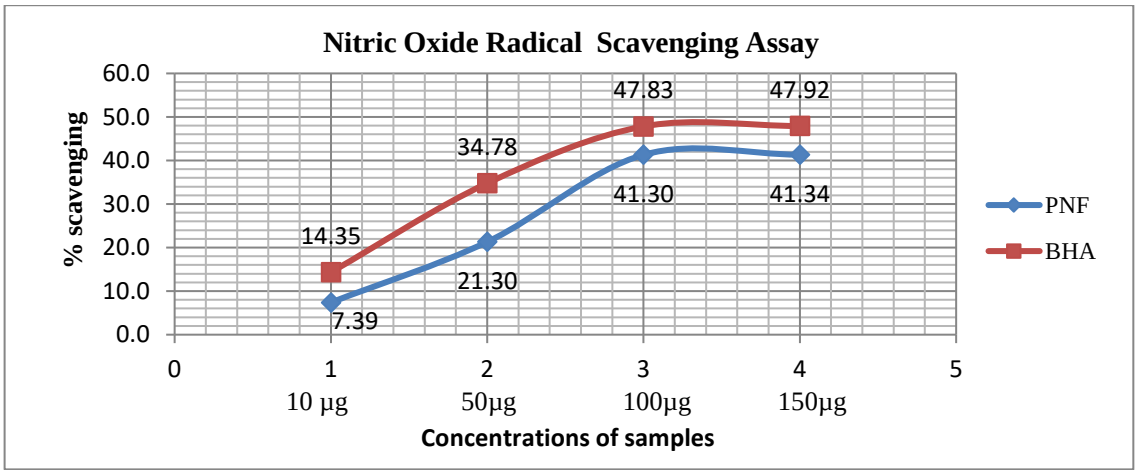


Fig. 2. Percentage nitric oxide free radical scavenging activity of test drugs such as PNF and BHA at different concentration

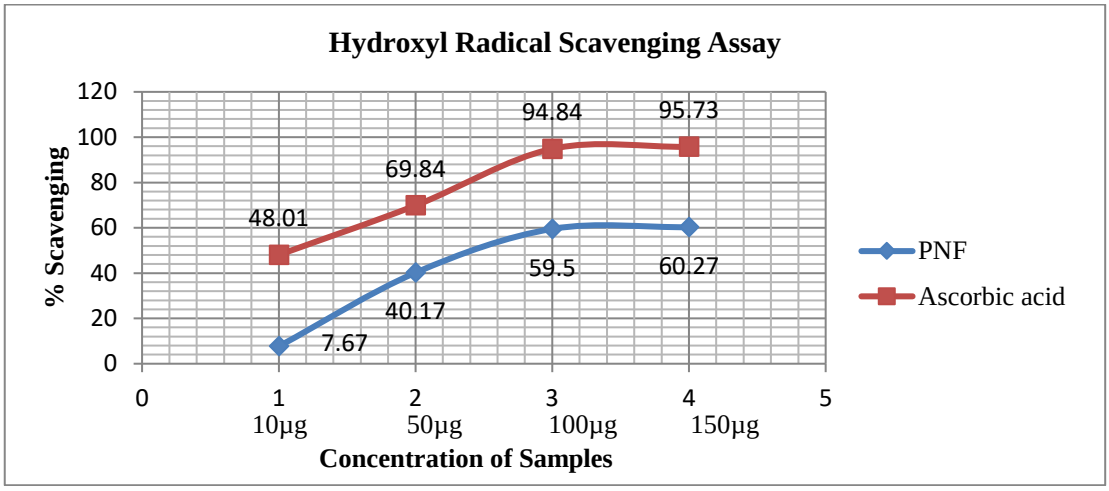


Fig. 3. Percentage hydroxyl free radical scavenging activity of test drugs such as PNF and ascorbic acid at different concentration

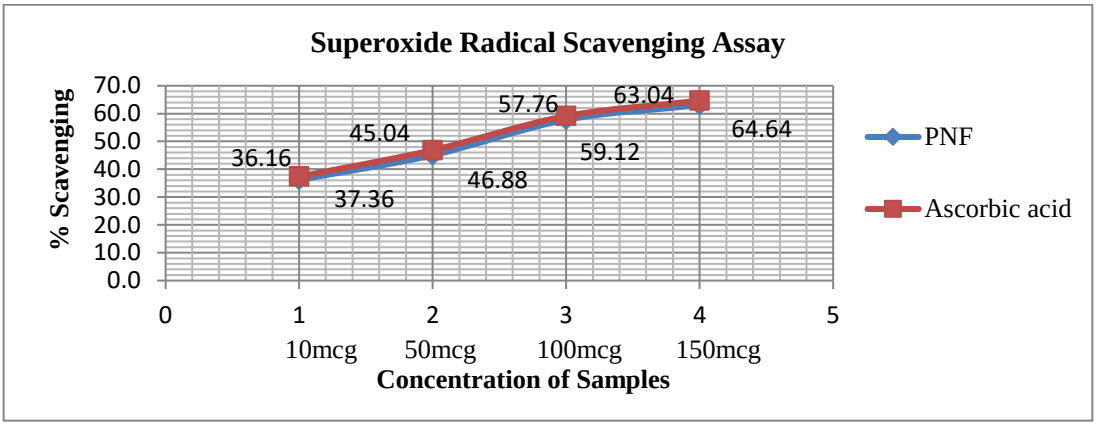


Fig. 4 Percentage superoxide free radical scavenging activity of test drugs such as PNF and ascorbic acid at different concentration

Superoxide radical scavenging activity

In this assay, superoxide anions were formed by using PMS-NADH-NBT system. In this assay, the capturing of superoxide anion and better free radical scavenging potential of the test compounds which were correlated with decrease in absorbance values when observed at 560 nm. The results obtained in this assay, showed that the IC_{50} values of test synthetic novel flavonoid (PNF) and Ascorbic acid (AA) were 2.44 and 2.28 μg/ml respectively, indicating better superoxide (O_2^{*-}) anion radical scavenging activity. In addition to this, these compounds exhibited dose-dependent O_2^{*-} scavenging activity. Fig.4 represents the percentage inhibition of superoxide radical generation at different concentrations of test PNF compound and standard ascorbic acid. PNF exhibited concentration-dependent scavenging activities against superoxide anion radicals generated in PMS-NADH systems. The test novel flavonoid (PNF) showed significant superoxide radical scavenging activity (63.04%), at 150 µg/mL concentration.

DISCUSSION

Capillary tube system of melting point and TLC methods were used to determine the purity of test synthesized compound (PNF). The synthesized compound was further established by IR, 1H NMR, and mass spectral studies. Based on spectral data, it was proved that the synthesized chalcone and flavone derivative meet the standard values of various spectral techniques. Free radicals are highly reactive molecules, associated with oxidative damage where as antioxidants are reducing agents, as they donate electrons to free radicals and preventing them from oxidative damage to biological structures. Due to the instability, insolubility and tedious standardization procedures, the natural flavonoids though highly potent and good antioxidant properties, they are not preferred now a days (11). Hence, study has been undertaken to synthesize the novel flavonoid in laboratory to determine its free radical scavenging antioxidant activities in *invitro* methods.

The diphenyl-picrylhydrazine is unstable, purple colored nitrogen radical and has strong absorption at 517 nm. In this DPPH assay, less stable DPPH radicals are reduced to the more stable yellow coloured DPPH in presence of antioxidants. This method is based on the fact that the hydrogen ion donation by antioxidants in the alcohol solution converts the free radicals such as DPPH to yellow colored non radical reduced form

DPPH-H (18). From the results the test novel flavonoid (PNF) exhibited the dose dependent DPPH scavenging activity this might be due to its proton donating property.

Nitric oxide, apart from its beneficial effects, is also involved in pathological conditions such as inflammation, cancer etc. Nitric oxide was generated from sodium nitroprusside and measured by Greiss reaction. Scavengers of nitric oxide compete with the oxygen, leads to decreased nitric oxide production. Presence of phenolic structure in the test flavonoids as said in previous research reports Hernández *et al*., (19) and Revathi and Rajeshwari (20) play a vital role in NO radical scavenging activity, which might be the reason behind differential inhibitory effect observed in this study. Nitric oxide radical scavenging property of the test PNF compound may be due to the electron donating nature of the substituent's OH and -CH_3 groups present in its benzopyran nucleus.

By the oxidation reaction with the dimethyl sulphoxide (DMSO), hydroxyl radicals were formed along with formaldehyde which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent (21). Results tabulated in table-3 suggests that the synthetic flavonoid(PNF) when used at different concentrations(10 µg/ml, 50 µg/ml and 100 µg/ml)shown to exhibit a dose dependent hydroxyl radical scavenging activity. The presence of hydrogen donating ability of phenolic groups in the test synthetic flavonoid is proposed to be responsible for free radical scavenging activity which supports the work of Pavithra and Vadivukkarasi (22).

In different biological systems, superoxide anions are the most common free radicals generated and under conditions of oxidative stress the concentration of these anions increases(23). Though these superoxide radicals have a weak oxidant, relatively less chemical reactivity, under oxidative stressful conditions, they can produce very dangerous singlet oxygen and hydroxyl radical reactive components which leads to lipid peroxidation(24). The flavonoids are often considered as antioxidants as they have property of donating charged molecules due to the presence of electron donating substituent groups like –OH, -CL and –CH_3 in their chemical benzopyran nucleus(25). Therefore these compounds donated their electrons to the superoxide and scavenge them to prevent their further interaction with NBT followed by inhibition of formation of blue colour formazan product. The presence of OH groups and methyl groups in their chemical structure may be responsible for their radical scavenging power of the test flavonoid(PNF) and standard drug. This action of these test and standard compounds may be explained due to their property of quenching the oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical or neutralize free radicals or by their chelating ability due to their high nucleophilic character of the aromatic ring.

CONCLUSION

From the present study, it is concluded that the test novel flavonoid (PNF) has a significant free radical scavenging activity as compared to standard drugs such as BHA and ascorbic acid. Further study is required to discover its molecular mode of action involved in antioxidant activity. Antioxidant potential of flavonoids may be the major role for their therapeutic implications in oxidative stress diseases such as diabetes, dyslipidemia, Alzheimer's disease etc.,

ACKNOWLEDGEMENT

The authors are thankful to BVVS's S. N Medical College Bagalkot and B. M. Patil Medical College, BLDE, Vijayapura, for providing the laboratory facilities.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

REFERENCES

1. Usha, B., Karanth, J.,Joshi,C.G.Antioxidant, anti-inflammatoryantioxidant, anti-inflammatory, analgesic and anti-proliferating activities of *Grewia heterotricha* Mast. Biomedicine, 2020; 40(3): 272- 277.
2. Majewska, M., Skrzycki, M., Podsiad, M., Czeczot, H. Evaluation of antioxidant potential of flavonoids: an*in vitro* study. Acta Pol Pharm, 2011;68(4):611-615.
3. Joyce, D.A. Oxygen radicals in disease. Adv. Drug React. Bull, 1987;127: 476-479.
4. Pham-Huy, L.A., He, H., Pham-Huy, C. Free radicals, antioxidants in disease and health. Int J Biomed Sci 2008; 4(2):89-96.
5. Potbhare, M., Khobragade, D.*In vitro*evaluation of antioxidant potential of ayurvedic preparationsLauhaBhasma and ManduraBhasma. Asian J. Pharm. Res. 2017; 7(2): 63-66.
6. Cross, C.E., Valacchi, G., Schock, B.,Wilson, M.,Weber, S.,Eiserich, J.,*et al*.,Environmental oxidant pollutant effects on biologic systems: a focus on micronutrient antioxidant-oxidant interactions. American Journal of Respiratory and Critical Care Medicine, 2002;166(1):S44-S50.
7. Yamagishi, S., Matsui, T. Nitric oxide, a Janus-faced therapeutic target for diabetic microangiopathy-Friend or foe? Pharmacological Research, 2011; 64: 187-194.
8. Muthukumaran, P., Abirami, C., Priyatharsini. Antioxidant and free radical scavenging activity azimatetracanthal. Am. Research J. Pharma. Dosage Forms and Tech. 2013; 5(6): 315-319.
9. Santos, C.M.M., Silva, A.M.S. The antioxidant activity of prenylflavonoids. Molecules. 2020; 25(3):696.
10. Chen, X., Mukwaya, E., Wong, M.S., Zhang,Y. A systematic review on biological activities of prenylated flavonoids. Pharm. Biol. 2014; 52: 655-660.
11. Vijay Kumar, D. Synthesis of some aryl and heteroaryl substituted γ-benzopyrones of biological interest. Ph D Thesis, Manipal University Manipal, Karnataka India. 2010.
12. Oyamada, T. A new general method for the synthesis of flavonol derivatives. J. Chem. Soc. Jpn 1934; 55: 1256-1261.
13. Nespor, J., Hanko, V., Karabín, M., Jelínek, L., Dostálek, P. Prenylated flavonoids as valuable biologically active compounds from hops. KvasnyPrum. 2017; 63: 164-172.

14. Klein, S. M., Cohen, G., Cederbaum, A. I. Production of formaldehyde during metabolism of dimethyl sulphoxide by hydroxyl radical generating system**.** Biochemistry, 1981; 20: 6006-6012.
15. Sun, J., Zhang, X., Broderick, M., Fein, H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors, 2003; 3:276-284.
16. Jia, Z.S., Tang, M.C, Wu, J.M. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 1999; 64(4):555-559.
17. Ozcelik,J.H, Lee,D.B. Min. Effects of Light, Oxygen, and pH on the Absorbance of 2,2-Diphenyl-1-picrylhydrazyl. Journal of Food Science, March 2003;68(2):487-490.
18. Olugbami, J.O., Gbadegesin, M.A., Odunola, O.A. *In vitro* free radical scavenging and antioxidant properties of ethanol extract of *Terminalia glaucescens*. Pharmacognosy Res. 2015;7(1):49-56.
19. Hernández, M., Ventura, J., Castro, C., Boone, V., Rojas, R., Ascacio-Valdés, J.,*et al.*,UPLC-ESI-QTOF-MS2-Based identification and antioxidant activity assessment of phenolic compounds from red corn cob (*Zea mays* L.). Molecules, 2018; 23(6):1425.
20. Revathi, D., Rajeswari, M. *In vitro*evaluation of nitric oxide scavenging activity of *Guettarda speciosa* Linn. International Journal of Science and Research (IJSR), September 2015;4(9):962-965.
21. Bora, K.S., Sharma, A. Evaluation of antioxidant and free-radical scavenging potential of *Artemisia absinthium*. Pharm Biol. 2011;49(12):1216-1223.
22. Pavithra, K.,Vadivukkarasi, S.Evaluation of free radical scavenging activity of various extracts of leaves from *Kedrostisfoetidissima*(Jacq.)Cogn.Food Science and Human Wellness, 2015;4:42-46.
23. Halliwell, B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radical Research. 1996;25:57-74.
24. Hayyan, M., Hashim, M. A., AlNashef, I. Superoxide Ion: Generation and chemical implications. Chemical Reviews. 2016; 116 (5), 3029-3085.
25. Wang, W.K., Park, H.S., Ham, I., Oh, M., Namkoong, H., Kim, H.K., *et al.*,Natural compounds, fraxin and chemically structurally related to fraxin protect cells from oxidative stress. Exp. Mol. Med. 2005; 37: 436-446.