Original Article

In vitro Study of Antioxidant and Antimalarial Activities of New Chromeno-Pyrano-Chromene Derivative

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ABSTRACT

A new molecule of 2-[3, 4 di hydroxy phenyl] 3, 5, 14-trihydroxy 13-oxo 2H-chromeno [$3^1 4^1$:5, 6] pyrano [2, 3-h] 4H-chromene 4-one was synthesized and screened. This new compound was synthesized by incorporating 4-chloro-3-formyl coumarin group with quercetin compound. In the present study the antioxidant and antimalarial activities of new 2-[3, 4 di hydroxy phenyl] 3, 5, 14-trihydroxy 13-oxo 2H-chromeno [$3^1 4^1$:5, 6] pyrano [2, 3-h] 4H-chromene 4-one molecule was studied. The Antioxidant activity was performed by 1, 1-diphenyl-2-picryl hydrazyl free radical scavenging activity, phosphomolybdenum method, reducing power method and scavenging of nitric oxide methods and the calculated IC_{50} values for new molecule and reference standard gallic acid were 1.77µg/ml, 0.13µg/ml respectively for DPPH method and 1.93µg/ml, 0.28µg/ml respectively for nitric oxide method. The antimalarial activity was performed by SYBR green I assay and by inhibition of β-hematin formation. These findings reveal that the new molecule 2-[3, 4 di hydroxy phenyl] 3, 5, 14-trihydroxy 13-oxo 2H-chromeno [$3^1 4^1$:5, 6] pyrano [2, 3-h] 4H-chromene 4-one has good antimalarial and antioxidant activities and could be an alternate candidate for the development of new biologically active compounds.

Keywords: Antioxidant, Antimalarial, Chromeno-pyrano-chromene.

INTRODUCTION

In general Malaria and oxidative stress are major health problems in the world. Malaria is a mosquito-borne infectious disease of the blood caused by the parasite, *Plasmodium sp*. It spreads through the bite of infected female *Anopheles* mosquito and is endemic in tropical and sub-tropical regions[1]. Recent reports suggest that generation of reactive oxygen species and associated oxidative stress play a crucial role in the development of systemic complications in malaria[2]. Malarial infection

decreases the levels of antioxidant enzymes and other anti-oxidants such as catalase, glutathione (GSH) peroxidase, super-oxide dismutase, albumin, glutathione, ascorbate and plasma tocopherol[3]. An antioxidant, acts as a reducing agent that donates electrons to the free radicals. They are capable of deactivating or stabilizing free radicals before they attack cells[4]. Recent developments in biomedical point to the involvement of free radicals in many diseases. Free radicals attack the unsaturated fatty acids in the biomembranes resulting in membrane fluidity. For these reasons antioxidants are of interest for the treatment of many kinds of cells degeneration[5].

The synthesized molecule contains Chromene (Benzopyran) moiety which is one of the privileged scaffold which appears as an important structural component in various natural products like flavonoids, coumarins and also possess useful phytochemical properties. The derivatives of Chromene moiety can be capable of interacting with a variety of cellular targets which leads to their wide ranging biological activities such as antitumor, anti-oxidant, anti-inflammatory, anti-coagulant, hypo-thermal, vasodilatory, anti-HIV, anti-tubercular, analgesic activity, antimalarial activity etc[6-8].

Therefore, this study is primarily designed to determine the antimalarial and antioxidant activities of the new molecule 2-[3, 4 di hydroxy phenyl] 3, 5, 14-trihydroxy 13-oxo 2H-chromeno [$3^1 4^1$:5, 6] pyrano [2, 3-h] 4H-chromene 4-one.

MATERIALS AND METHODS

In vitro anti-oxidant activity

Chemicals and reagents

All the chemicals used were of Analytical grade. Gallic acid (gifted sample), DPPH (purchased from research-labfinechem industries, Mumbai), Methanol, Ethanol, Trichloroacetic acid, Phosphate buffer, Ammonium molybdate, Sodium phosphate, DMSO, Sodium nitro prusside.

DPPH (1, 1-diphenyl -2-picryl hydrazyl) free radical scavenging activity

The molecule 1, 1-diphenyl-2-picryl hydrazyl (α, α-diphenyl-βpicrylhydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electrons also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of violet color. In order to evaluate the antioxidant potential through free radical scavenging by the test sample, the change in optical density of DPPH radicals is monitored[4,9].

The free radical scavenging activity of the synthesized compound was measured by decrease in absorbance of the methanolic solution of DPPH. 0.1mM solution of DPPH in methanol was prepared. Gallic acid was taken as reference standard. Different concentrations of test sample (5µg/ml, 10µg/ml, 20µg/ml, 40µg/ml) and standard (1.0µg/ml, 2.5 µg/ml, 5.0 µg/ml) were prepared using ethanol. 1.0 ml of 0.1mM DPPH solution was added to 3.0 ml of all concentrations of test and standard separately. These solutions were kept in the dark for about 30min and the absorbances were measured at 517nm. Ethanol (3 ml) in the place of test sample was used as the blank. The capability to scavenge the DPPH radical was calculated using the following equation[10].

DPPH Scavenged (%) = $\{(A_0-A_1)/A_0\} \times 100$.

Where: A_0 is the absorbance of the blank (containing all reagents except the test sample), and A_1 is the absorbance of the test sample. The antioxidant activity of test sample was expressed as IC_{50}. The IC_{50} value is defined as the concentration in (µg/ml) of test sample that scavenges free radicals by 50%.

Phosphomolybdenum assay

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH[4].

Different concentrations of test sample (5µg/ml, 10µg/ml, 20µg/ml, 40µg/ml) and standard (1.0µg/ml, 2.5µg/ml, 5.0µg/ml) Gallic acid were prepared using a suitable solvent. 0.3ml of each concentration of test sample and the standard was mixed with 3.0ml of reagent (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were capped and incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695nm using UV-visible spectrophotometer after cooling to room temperature. Distilled water (0.3 ml) was used as blank in place of test sample[11].

Nitric oxide generation & assay of nitric oxide scavenging

NO is generated in biological tissues by specific nitric oxide synthases, that metabolizes arginine to citrulline with the formation of NO via a five electron oxidative reaction. The compound sodium nitroprusside decompose in aqueous solution at physiological pH (7.2) producing NO under aerobic conditions. NO reacts with oxygen to produce stable products (nitrate and nitrite), the quantities of which can be determined using Griess reagent[4].

Sodium nitroprusside (5mM) in phosphate buffered saline was mixed with different concentrations of test sample (5µg/ml, 10µg/ml, 20µg/ml, 40µg/ml) dissolved in Ethanol & incubated at 25°C for 150 min. The samples above were reacted with Greiss reagent (1% sulphanilamide, 2% ortho phosphoric acid & 0.1% naphthylethylenediamine dihydrochloride).

The absorbance of the chromophore formed during the naphthylethylenediamine was read at 546nm & referred to the absorbance of standard solutions of Gallic acid was treated in the same way with Greiss reagent. All the tests were performed in triplicate & the results was averaged. Gallic acid was used as reference standard. The % inhibition in absorbance was calculated[11].

Nitric oxide Scavenged (%) = $\{(A_0-A_1)/A_0\} \times 100$.

Where A_0 is the absorbance of the blank (containing all reagents except the test sample), and A_1 is the absorbance of the test sample.

Reducing power method

This method is based on the principle of increase in the absorbance of the reaction mixtures. An increase in the absorbance indicates an increase in the antioxidant activity. In this method, an antioxidant compound forms a colored complex with potassium ferricyanide, Trichloroacetic acid and ferric chloride, which is measured at 700 nm. Increase in absorbance of the reaction mixture indicates the reducing power of the sample[4].

In this method, 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v) are added to 1.0 ml of test sample (5µg/ml, 10µg/ml, 20µg/ml, 40µg/ml) and standard (1.0µg/ml, 2.5µg/ml, 5.0µg/ml) Gallic acid dissolved in distilled water. The resulting mixture is incubated at 50°C for 20 min, followed by the addition of 2.5 ml of Trichloroacetic acid (10% w/v).

The mixture is centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 ml), mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v). The absorbance is then measured at 700 nm against blank sample[12,13].

In vitro antimalarial activity

Procedure

Assessment of *in vitro* anti-malarial activity of test sample using SYBR green I assay

The antimalarial activity of test sample was investigated by using SYBR Green I assay[14,15]. Highly synchronous ring stage parasite was used in each assay. An aliquot of parasite inoculum (50µl) with 2% parasitaemia and 1% haematocrit was added into each well of microtiter plate. Test sample was added to the malaria culture at various concentrations (1.5-90nM). Chloro-quine (3.89-498.15nM) and artesunate (0.39-50.0nM) were used as standard anti-malarial drugs. The experiment was conducted in triplicate. IC_{50} values (drug concentration that inhibits the parasite growth by 50%) were used as an indicator of anti-malarial activity and was determined from a log dose–response curve plotted using the Calcusyn™ version (BioSoft, Cambridge, UK).

Evaluation of *in vitro* inhibition of β-hematin formation by test sample

The ability of the test sample to inhibit β-hematin formation was induced by 1-oleoyl-rac-glycerol using a UV spectrophotometer and measurements were carried out at 405 nm[16]. The 50% inhibitory concentration (IC_{50}) values of the Test Sample was obtained from the sigmoidal dose response curves using non-linear regression curve fitting analysis with GraphPad Prism v.4.03 software.

Statistical analysis

The experimental data were expressed as mean ± SEM and the IC_{50} values are calculated by using graph Pad Prism 6 software.

RESULTS AND DISCUSSION

The results of antioxidant values expressed as IC_{50} against various free radicals shown in Table 1 and Table 2. The calculated IC_{50} values for synthesizing chromeno-pyrano-chromene derivative and reference standard Gallic acid were 1.77µg/ml, 0.13µg/ml, respectively for DPPH method and 1.93µg/ml, 0.28µg/ml respectively for the nitric oxide method. The free radical scavenging activity of the compound is concentration dependent, as the concentration of the test compounds increases, the radical scavenging activity increases and lower IC_{50} value reflects better protective action[4,11]. The antioxidant activity of the synthesized chromeno-pyrano-chromene derivative could be attributed to electron donating nature of the substituents like –OH, on the scaffold, reduce free radicals and prevent the damage of cells. The more the hydrogen donors, the stronger is the anti-oxidant activity[6].

The antimalarial activity of the compound was carried out. From the present study it was proved that chromeno-pyrano-chromene derivative had shown good antimalarial activity when compared to that of standard chloroquine and artesunate drugs. The results were shown in Table 3, 4. The presence of Chromene nucleus and heterocyclic ring system may be responsible for anti-oxidant and anti-malarial activity.

CONCLUSION

Results of the present study demonstrate that, new compound 2-[3, 4 di hydroxy phenyl] 3, 5, 14-trihydroxy 13-oxo 2*H*-chromeno [$3^1 4^1$:5, 6] pyrano [2, 3-h] 4*H*-

chromene 4-one possessed good *in vitro* antioxidant, antimalarial activities. Owing to these aspects this new compound need to be given much attention in medicinal research to establish its biological efficacy as an antimalarial agent.

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REFERENCES

1. Andrej Trampuz[1], Matjaz Jereb[2], Igor Muzlovic[3] and Rajesh M Prabhu[4]. Severe malaria. *Clin Rev* 2003; 7 (4): 315-323.
2. Pabon A, Carmona J, Burgos LC and Blair S. Oxidative stress in patients with non-complicated malaria. *Clin Biochem* 2003; 36:71-78.
3. Clark IA, Chaudhri C and Cowden WB. Some roles of free radicals in malaria. *Free Radic Biol and Med* 1989; 6:315-321.
4. Md. Nur Alam*, Nusrat Jahan Bristi, Md. Rafiquzzaman. Review on in vivo and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal* 2013; 21:143–152.
5. Halliwell B, Aeschbach R, Lolliger J and Aruoma OI. The characterization of antioxidants. *Food Chem Toxicol* 1995; 33:601-617.
6. Nancy thomas and Subinmary Zachariah*. Pharmacological Activities of Chromene Derivatives; an overview. *Asian Journal of Pharmaceutical and Clinical Research* 2013; Vol 6: Suppl 2, ISSN - 0974-244.
7. Moon HI[1], Lee JH, Lee YC, Kim KS. Antiplasmodial and cytotoxic activity of coumarin derivatives from dried roots of Angelica gigas Nakai *in vitro*. *Immunopharmacol Immunotoxicol.* 2011; 33(4):663-6.
8. Ganesh D[1], Fuehrer HP, Starzengrüber P, Swoboda P, Khan WA, Reismann JA, Mueller MS, Chiba P, Noedl H. Antiplasmodial activity of flavonol quercetin and its analogues in Plasmodium falciparum: evidence from clinical isolates in Bangladesh and standardized parasite clones. *Parasitol Res* 2012; 110(6): 2289-95.
9. Ilango. K[1] & Renjith Alex. A*[2]. Phytochemical Screening and Evaluation of *In vitro* Antioxidant Activity of Viburnum punctatum buch-ham. Ex d.don. *AJPCT* 2013*;* 1[4]: 416-423.
10. Sharma A, Bhardwaj S, Mann A.S, Jain A. and Kharya M.D. Screening methods of antioxidant activity: an overview. *Pharmacognosy Reviews* 2007; Vol. 1(2): 232-238.
11. K. V. Ratnam, K. Ravishankar and P. Priyabhandavi. Evaluation of *in vitro* antioxidant activity of ethanolic root extract of curculigoorchioides. *IJRPC* 2013; 3(2): 2231-2781.
12. Oyaizu M. studies on product on browning reaction prepared from glucose amine. *J. pn. J. Nutr* 1986; vol.44: 307-315.
13. Swagata Pal[1], Ankita Bhattacharjee[2], Sandip Mukherjee[2], Koushik Bhattacharya[2] and Suman Khowala*[1]. Antioxidant and Hepatoprotective Activity of Ethanolic Extract of Alocasia indica Tuber, *AJPCT* 2014; 2: 191-208.
14. Lambros C, Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* 1979; 65:418–420.
15. Bennett TN, Paguio M, Gligorijevic B, Seudieu C, Kosar AD, Davidson E, Roepe PD. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrob Agents Chemother* 2004; 48:1807–1810.
16. A.K. Tripathi, S.I. Khan, L.A. Walker, B.L. Tekwani. Spectrophotometric determination of de novo hemozoin/b-hematin formation in an *in vitro* assay. *Anal. Biochem* 2004; 325, 85e91.

Table 1. DPPH, nitric oxide methods of test sample and gallic acid

		DPPH method		Nitric oxide method	
Sample	Concentration (µg/ml)	% Inhibition ± SEM	IC_{50} (µg/ml)	% Inhibition ± SEM	IC_{50} (µg/ml)
Test Compound	5 10 20 40	63.9±0.635 71.1±0.808 80.0±0.412 83.92±0.204	1.77	68.0±0.047 74.6±0.221 85.09±0.109 91.6±0.140	1.93
Gallic Acid	1.0 2.5 5.0	79.2±0.523 87.1±0.146 92.0±0.248	0.13	74±0.426 85±0.066 92±0.051	0.28

Values are expressed as mean ± SEM, n=3

Table 2. Phosphomolybdenum and reducing power method of test sample and gallic acid

		Phosphomolybdenum method	Reducing power method
Sample	Concentration (µg/ml)	Absorbance ± SEM	Absorbance ± SEM
Test Compound	5 10 20 40	0.088±0.0008 0.129±0.001 0.154±0.001 0.185±0.001	0.046±0.011 0.061±0.02 0.074±0.011 0.089±0.011
Gallic Acid	1.0 2.5 5.0	0.0145±0.0001 0.017±0.0002 0.036±0.0001	0.053±0.002 0.136±0.003 0.165±0.005

Values are expressed as mean ± SEM, n=3

Table 3. *In vitro* anti-malarial activity of test sample, chloroquine and artesunate

Sample	IC_{50} against chloroquine-sensitive *Plasmodium falciparum* (nM)	IC_{50} against chloroquine resistant *Plasmodium falciparum* (nM)
Artesunate	5.12 5.98 4.77	3.54 3.82 2.99
Chloroquine	14.3 16.92 15.44	247.45 269.39 285.34
Test sample	18.05 19.2 17.32	59.22 63.87 56.41

Table 4. *In vitro* inhibition of β-hematin formation

Sample	IC_{50} (µM)
Artesunate	21.18 22.68 20.71
Chloroquine	73.3 77.92 85.67
Test sample	21.24 19.45 18.29

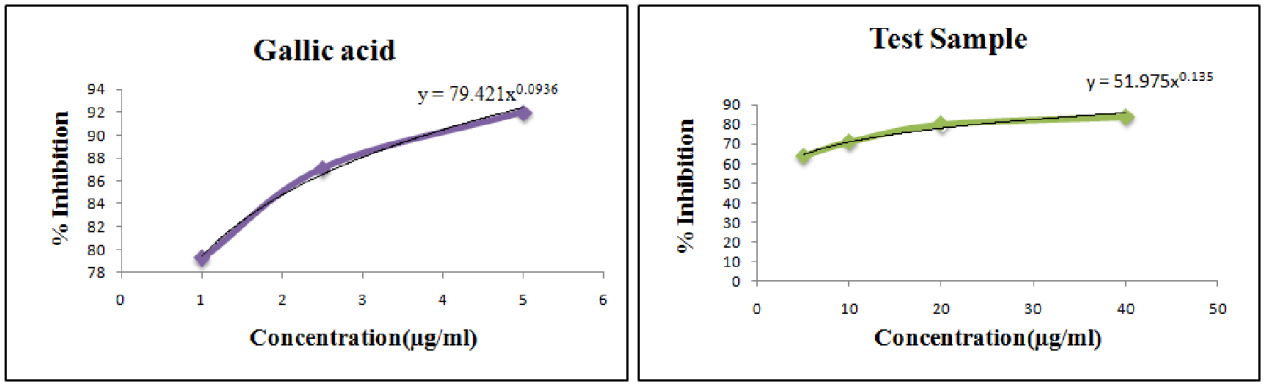

Figure 1. Graphs for DPPH method

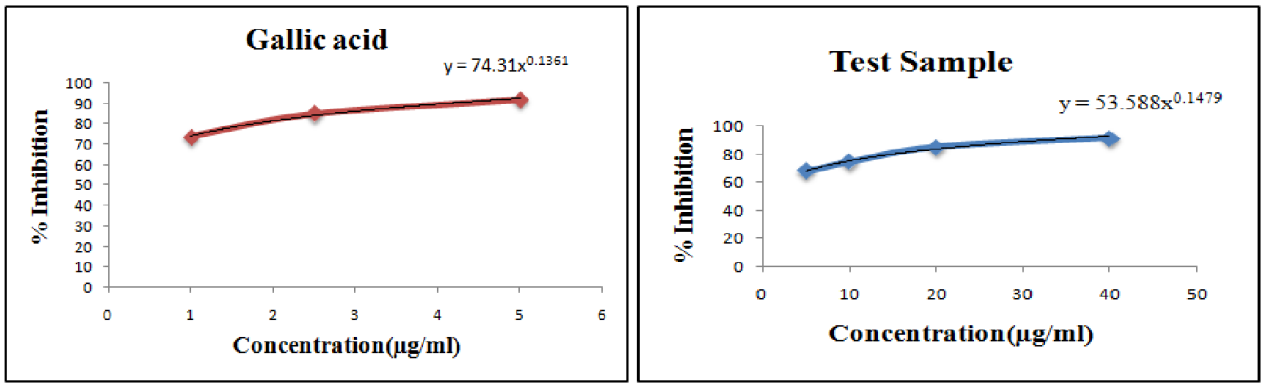

Figure 2. Graphs for nitric oxide method

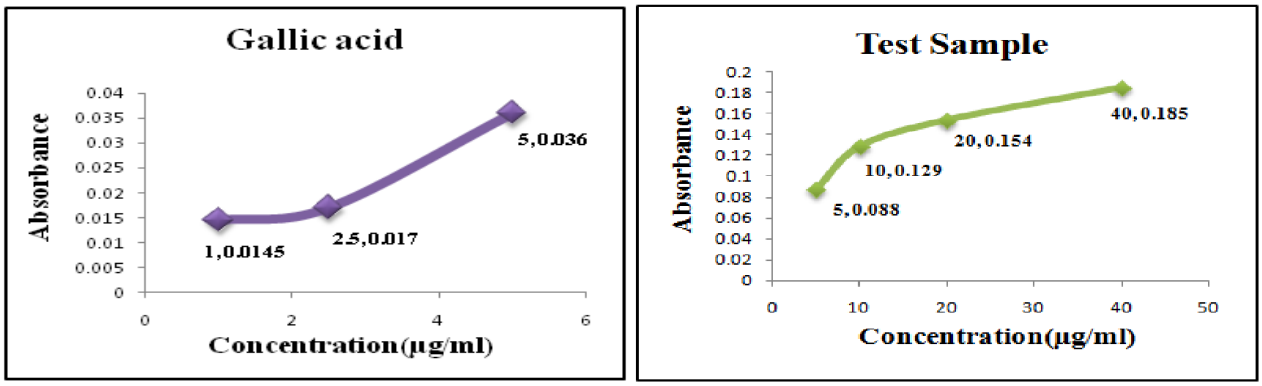

Figure 3. Graphs for phosphomolybdenum method

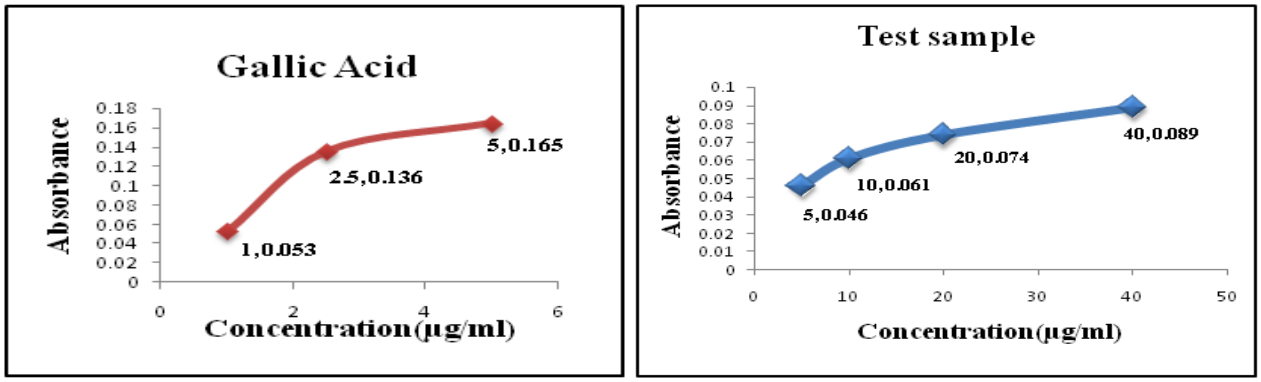

Figure 4. Graphs for Reducing power method