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ARTICLE

Pharmacological Investigation of Fluoro, Iodo and Hydroxy Derivative of Chloro Substituted Homoisoflavonoids

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Asian Journal of Organic & Medicinal Chemistry

Volume: 7 Year: 2022
Issue: 1 Month: January–March
pp: 31–36
DOI: https://doi.org/10.14233/ajomc.2022.AJOMC-P357

Received: 28 September 2021
Accepted: 23 February 2022
Published: 5 April 2022

ABSTRACT

In present study, the synthesized substituted homoisoflavonoid derivatives were screened for their *in vivo/in vitro* antiarthritic activity, *in vitro* anti-inflammatory and DPPH free radical scavenging activity. The male Wistar rats were used for investigation of *in vivo* antiarthritic activity against complete freund's adjuvant (CFA) induced arthritis and assessment was done for change in paw volume, serum marker enzymes (ALP, SGOT and SGPT) and membrane stabilization potential. *in vitro* anti-inflammatory activity was assessed by the protein denaturation method. *In vitro* free radical scavenging activity was assessed by the DPPH method. The result indicated that compound **HIFa** showed a significant antiarthritic activity as compared to other substituted homoisoflavonoid derivatives. The significant membrane stabilization and inhibition of protein denaturation showed *in vitro* antiarthritic and anti-inflammatory activity of substituted homoisoflavonoid derivatives. The substituted homoisoflavonoid derivatives showed dose dependent DPPH free radical scavenging activity. From the present study, it was observed that the iodo derivative of substituted homoisoflavonoid derivatives have significant pharmacological activities as compared to floro and hydroxyl derivatives.

KEYWORDS

Homoisoflavonoid derivatives, Anti-inflammatory activity, DPPH assay, Antiarthritic activity, Homoisoflavonoid derivatives.

INTRODUCTION

Flavonoids and their subclass like homoisoflavonoid are the recent areas of study to explore their potential role in plants and human beings for several purposes including medicinal uses. They are especially occurring in *Fabaceae* and *Asparagaceae* families and rarely in *Polygonaceae*, *Portulacaceae*, *Orchidaceae*, *Gentianaceae*, *etc.* [1]. The homoisoflavonoid occurs in the number of plant families and is a very important class of secondary metabolites. The presence of homoisoflavonoid has been identified and confirmed from different parts of the plants like roots, barks, heartwood, bulbs, leaves and seeds, *etc.* which are been using as folk medicines for several clinical alignments [1].

Arthritis is a commonly occurring joint disease amongst the population of developing and developed countries particularly in aged persons [2]. It has been reported that joint related diseases are highly contributing to compensate physical

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disabilities, decreased work capabilities and influencing the day to day performance [3]. India is also having a huge burden of arthritic patients and numbers are quite worrying. Studies have revealed and reported that nearly 45% of women above the age of 65 years have symptoms of arthritis [4] and the percentages of women are higher as compare to males [5]. The common symptoms of arthritis are pain, inflammations, swelling, redness and stiffness, which cause patients to seek pharmacotherapy and medical advice [6]. The current pharmacotherapy involves chronic treatment of NSAIDs, corticosteroids, opioid analgesics and DMARDs [7]. However chronic consumption of the above drugs leads to several severe side effects like hepatotoxicity, nephrotoxicity, hormonal imbalance, acquisition of infection, disturbed water and electrolyte balance, drug dependence, *etc.* which limits their use beyond certain doses, duration and patient-related factors [8].

To date, several homoisoflavonoids have been reported and their numbers are more than 300 [9]. The systemic understanding of homoisoflavonoid is classified or organized in five different groups as sappanin-type (I), scillascillin-type (II), brazilin-type (III), caesalpin-type (IV) and protosappanin-type (V). Ferreirin and homoferreirin were the first representative compounds from the class of isoflavonoids. Homoisoflavonoid is known as 3-benzyl-4-chromenones or 3-benzylidine [10]. Several studies have studies homoisoflavonoid and reported different biological activities. The spectrum of activities includes anti-inflammatory, antidiabetic, antioxidant, antimicrobial, antimutagenic, antioxidant, immunomodulatory, cytotoxic, antiangiogenic, vasorelaxant effects, *etc.* [11-13].

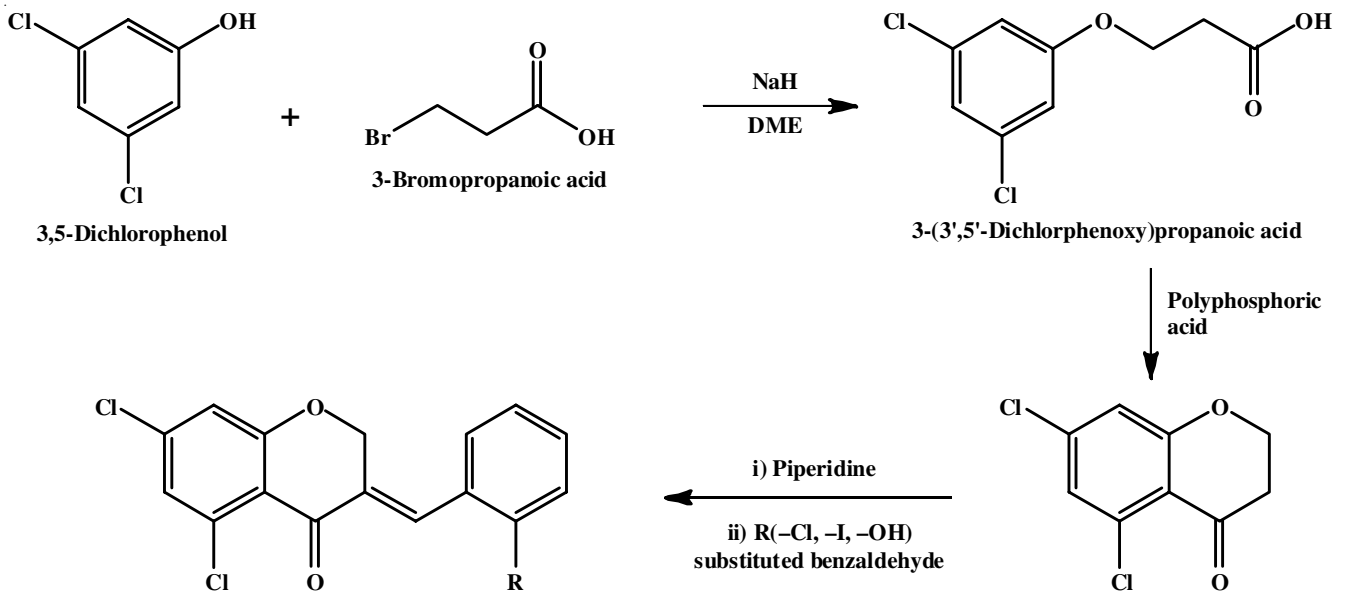
EXPERIMENTAL

All the chemicals used for the synthesis were of analytical grade and obtained from Sigma-Aldrich and S.D. Fine Chemicals, Mumbai, India. The purity of compounds was checked using TLC (0.25 mm silica gel plates) obtained from Merck India and observed under UV lights. The characterization of compounds was carried out using IR, NMR, UV-visible spectroscopy and mass spectrometry.

Synthesis of homoisoflavonoids: In step 1, compound 3-(3,5-dichlorophenoxy)propanoic acid was synthesized from 3,5-dichlorophenol by the action of NaH and 3-bromopropanoic acid in DMF. Further in step 2, 3-(3,5-dichlorophenoxy)-propanoic acid was treated with polyphosphoric acid to give 5,7-dichloro-2,3-tetrahydro-4*H*-chromen-4-one. Step 3 involves the synthesis of isoflavonoid derivatives of 2-fluorobenzaldehyde/2-iodobenzaldehyde/2-hydroxybenzadehyde from substituted benzaldehyde and 5,7-dichloro-2,3-tetrahydro-4*H*-chromen-4-one with piperidine (**Scheme-I**).

Acute toxicity studies and cell viability assay: To determine the safe dose of homoisoflavonoid derivatives in the animals, adult albino mice (Swiss strain) of either sex weighing between 25-30 g were used. The derivatives were administrated orally in various doses levels like (500, 750, 1000, 1500, 1750 and 2000) mg/Kg body. The test compounds (in different concentrations) were prepared in 0.5% sodium CMC were administered orally as a single dose in two mice, respectively. The behaviour changes, toxic reactions and mortality were observed in the animal for 72 h [14]. The cell viability assay was carried out for all the derivatives to determine the non-toxic and safe concentration to evaluate the *in vitro* anti-inflammatory activity. The toxicity was compared against lipopolysaccharides treated with cells. The safe concentration was considered to those compounds that showed cell viability above 90%.

Proposed pharmacological activity of homoisoflavonoids was carried out using adult healthy male Wistar rats weighing between 150-200 g for the experiment procured for an authorized animal vendor. The animals were maintained at standard laboratory conditions *viz.* temperature, hygiene, humidity and were monitored during the treatment protocol. Acclimation periods of 15 to 20 days were kept before the beginning of the experimental study. Animals were checked daily for any



Scheme-I: Synthesis of fluoro, iodo and hydroxy derivative of chloro-substituted HIF's

presence of infection/disease during the study. Animals were had free access to food and water with ad *libitum*. All the CPCSEA guidelines were followed during the experimental protocol and IAEC committee approval was taken before commencement of study.

Preparation of test and standard drug: The suspension of homoisoflavonoid derivatives was prepared in 2% acacia for oral administration. The dose of the test sample was selected as 100 mg/kg (low dose) and 200 mg/kg (high dose). The indomethacin was used as standard drug and prepared in 1% acacia in a dose of 30 mg/Kg orally.

Preparation of CFA for induction of arthritis: The CFA was obtained from Sigma-Aldrich and administrated as per the instructions provided by the manufacturer. The preparation contains 1 mg of heat-killed and dried *M. tuberculosis* in 0.85 mL paraffin oil and 0.15 mL of mannide monooleate. CFA is mixed well with the normal saline with equal volume of adjuvant and shake it vigorously. The CFA concentration was 1 mg/mL and volume of 0.2 mL in rat was used to produce the inflammation. The tuberculin injection was used to administer the CFA more preciously [15]. The formed emulsion was kept at temperature of 2-8 ºC.

***in vivo* antiarthritic activity:** After an acclimation of 15 days period the animals were divided randomly into 9 groups containing 6 animals in each. The control group received vehicle only, arthritic control (CFA treated) group were administered 0.2 mL of CFA intra-planetary, standard treated animals were administered with 0.2 mL of CFA intra-planetary and 30 mg/Kg indomethacin orally. Test group animals are designated as homoisoflavonoids (**HIFa and HIFc**) were administered with 0.2 mL of CFA intraplanetary with oral administration of respective test compounds in two doses as 100 mg/Kg and 200 mg/Kg. The administration of CFA was carried out at 9 a.m. the morning and observations were recorded after 1, 2, 4, 8, 16 and 24 h. The CFA was injected with 1 mL tuberculin syringe. The volume for CFA didn't exceed more than 0.2 mL. The change in paw volume was recorded as an index of antiarthritic activity. The change in paw volume was measured using a mercury plethysmometer. To assess the change in paw volume initially the circular mark was made on the right hind paw at the tibiotarsal junction. The mark helps to have the same dipping of the leg and ensure exact paw volume. Before any treatment, the right hind paw was dipped in mercury which was set initially 0 and change in mercury volume was noted [16,17].

***in vitro* antiarthritic activity:** Human red blood cell (RBC) is the commonly used method to screen the *in vitro* antiarthritic activity of test compound. The principle involves as the RBC membrane is similar to the lysosomal membranes. The stabilization of RBC in hypotonic solution correlated with stabilization of lysosomal membrane and implies the anti-inflammatory activity of test compounds [18]. The test mixture contains 1 mL phosphate buffer [pH 7.4, 0.15 M], 2 mL hypo saline (0.36%), 0.5 mL HRBC suspension (10% v/v) with 0.5 mL of test compound in different concentration (50, 100, 200, 500, 1000 µg/mL). Diclofenac sodium was used as a standard drug in different concentration (50, 100, 200, 500, 1000 µg/mL). The control samples consist of distilled water instead of hypo saline to produce 100% hemolysis. The samples were subjected for incubation at 37 ºC for 30 min and further centrifuged. From the suspension the hemoglobin content was estimated using spectrophotometer at 560 nm. The percentage hemolysis produced in the presence of distilled water was taken as 100%. Percentage of HRBC membrane stabilization or protection was calculated using the following formula [19]:

$$\text{Stabilization } (\%) = 100 - \frac{\text{Optical density of test solution}}{\text{Optical density of control}} \times 100$$

Estimation of serum marker enzymes: The blood was collected from the eye (orbital plexus) as a frequent and convenient way for sufficient amount of blood. Blood sample was collected by puncturing the retro-orbital plexus after 24 h that is after recording of physical parameters. About 1 mL of blood sample was collected in the clean and sterilized in effendroff's tubes and kept aside for clotting. The collected blood sample was proceeded for was centrifugation about 15 min at 3000 g. The serum was separated using micropipette and kept in the refrigerator for further investigation of serum marker enzymes *viz.* ALP, SGOT and SGPT estimation was carried out by using semi-autoanalyzer as per the procedure given by the manufacturer.

***in vitro* anti-inflammatory activity:** The method was used to investigate the anti-inflammatory effect of homoisoflavonoid derivatives in different concentration using the reported studies. The total volume of 1 mL test/standard solution (aqueous) at different concentration *viz.* (50, 100, 200, 500, 1000 µg/mL) was mixed with 1 mL of aqueous solution of bovine serum albumin (5%) and homogenized for 5 min and incubated at 27 ºC for 15 min. The control solution contains a equal volume *i.e.* 1 mL of water and 1 mL of bovine serum albumin (5%) was used. The protein denaturation was induced by placing the tubes in water bath maintained at 70 ºC for 10 min. After the same the mixtures were cooled at room temperature and absorbance was recorded at 660 nm using UV-visible spectrophotometry. Each observation was read for 3 times and mean of it was used for the calculation of inhibition of protein denaturation using following formula [20]:

$$\text{Protein denaturation inhibition } (\%) = \frac{A_{\text{control}} - A_{\text{treated}}}{A_{\text{treated}}} \times 100$$

The control represents 100% protein denaturation. The results were compared with acetyl salicylic acid (250 mcg/mL) treated samples.

DPPH radical scavenging activities: The DPPH was used to investigates the radical scavenging activity of homoisoflavonoid derivatives according to the reported methods [21]. Briefly the method involves different concentrations of test compound (50, 100, 200, 400 and 500 µg/mL) prepared in methanol. Ascorbic acid is used as standard drug prepared in methanol in same concentration as that of test compounds. The test sample contains 1 mL of test compound with 0.5 mL of 0.3 mM DPPH in methanol mixed well and kept for 15 min in dark place. Blank sample contains test compound and 1 mL of ethanol used to read to the baseline observation. The negative control contains 2.5 mL of DPPH solution and 1 mL of methanol, whereas ascorbic acid with different concentration was used as a positive/standard control. The observations were read at

517 nm using a spectrophotometer in triplicates and mean was used to determine the free radical scavenging activity using following eqn:

$$\text{Radical scavenging activity } (\%) = \frac{A_{control} - A_{standard}}{A_{control}} \times 100$$

RESULTS AND DISCUSSION

Homoisoflavonoids are new chemical entities emerging with diverse dimensions and applications [10]. We have synthesized new homoisoflavonoid derivatives and screened their biological potential. Induction of arthritis in animals can be produced by administration of monosodium iodoacetate, fluoroquinolones, lipopolysaccharides and CFA [22]. Amongst them, CFA-induced arthritis is a commonly used method for screening of antiarthritic activity. Administration of CFA leads to activation of immune cells and initiated inflammatory responses. The inflammatory mediators like interleukins, TNF-alpha are importantly found to increase in the animals [23].

Acute toxicity and cell viability: As the mortality was not observed at dose of 2000 mg/kg the 1/10th of dose that is 200 mg/kg as a high dose and 100 mg as a low dose was selected for the screening of biological activities [14]. According to the findings most of tested compounds were found safe at 100 µg dose level where are three compounds showed cell viability below 90% hence didn't considered for further evaluations.

Effect of homoisoflavonoid derivatives against CFI induced *in vivo* antiarthritis: In this study, it is found that administration of CFA causes an increase in paw volume when compared to the non-treated/control group. The swelling is occurred due to an increase in membrane permeability and enhanced fluid accumulation. This cascade is due to an increase in levels of pro-inflammatory mediators [24]. Treatment with homoisoflavonoid particularity 2-iodobenzaldehyde derivative showed the maximum inhibition of swelling, which is directly proportional to decrease in paw volume. The control group animal's shows the normal paw volume, administration of CFA causes significant ($p < 0.05$) increased in paw volume reported after 4 h of administration. The group of animals treated with homoisoflavonoids did not showed any significant decreased in paw volume compared against CFA treated animals for initial 1, 2 and 3rd h, however at 4 h of administration **HIFa1** and **b2** showed dose dependant significant decreased in paw volume ($p < 0.05$) when compared with CFA treated animals. Amongst the synthesized homoisoflavonoid derivatives, **HIFc** derivatives are less significant as compare to **HIFa** and **HIFb**. Paw volume readings at 8 h of treatment protocol CFA showed maximum paw swelling and gradually decreased due to its acute reactions (Fig. 1).

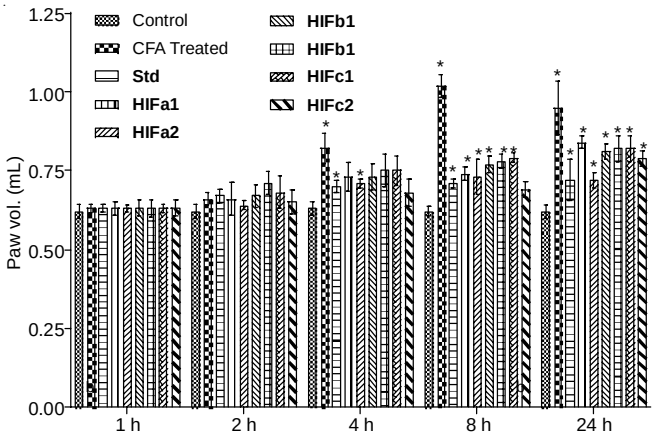


Fig. 1. Effect of HIF against CFI induced *in vivo* antiarthritis; Values are expressed as mean ± SD, n = 6, * is $p < 0.05$ is considered as significant, comparison was made as (control group *vs.* CFA treated group), (CFA treated group CFA + std.), (CFA treated group *vs.* CFA + **HIFa1**), (CFA treated group *vs.* CFA + **HIFa2**), (CFA treated group *vs.* CFA + **HIFb1**), (CFA treated group *vs.* CFA + **HIFb2**), (CFA treated group *vs.* CFA + **HIFc1**), (CFA treated group *vs.* CFA + **HIFc2**)

Effect of homoisoflavonoids on *in vitro* antiarthritic activity membrane stabilization: The *in vitro* investigation of antiarthritic activity can be evaluated by stabilization of membrane. The principle involved as the Human RBC membrane is comparable to the lysosomal membrane and its stabilization indicates that the test compound may also stabilizes the same. Stabilization of lysosomal is significant in limiting the inflammatory response by ceasing the release of lysosomal constituents of activated neutrophils which are important causes in the initiation of inflammatory cascades [25]. Homoisoflavonoid showed significant membrane stabilization and antiarthritic activity. The inhibition of hypotonicity induced HRBC membrane lysis *i.e.*, stabilization of HRBC membrane was taken as a measure of the anti-inflammatory activity. The percentage of membrane stabilization for homoisoflavonoids and standard drug were estimated for the different concentration of 50, 100, 250, 500, 1000 µg/mL. Compound **HIFa** showed effectively inhibiting the hypotonicity induced hemolysis of HRBC at dose dependent concentrations as shown below. It showed the maximum inhibition 33.44 ± 2.01 at 1000 µg/mL was reported for compound **HIFa**. With the increasing concentration the membrane hemolysis is decreased, whereas compounds **HIFb** and **HIFc** are comparatively less effective (Table-1).

Effect of homoisoflavonoid on serum marker enzymes: Several marker enzymes have been identified to diagnose the disease conditions, amongst them ALP, SGOT and SGPT are commonly evaluated to screen the antiarthritic activity of investigation compounds. These marker enzymes are found to

TABLE-1
EFFECT OF HIF PERCENTAGE OF HRBC MEMBRANE STABILIZATION

Treatment groups	50 µg/mL	100 µg/mL	200 µg/mL	500 µg/mL	1000 µg/mL
Standard	9.12 ± 2.50	17.35 ± 1.88	30.02 ± 2.80	55.66 ± 2.35	76.12 ± 3.11
HIFa	3.10 ± 0.77	5.05 ± 1.02	9.12 ± 1.10	15.45 ± 1.67	33.44 ± 2.01
HIFb	2.55 ± 0.62	3.95 ± 0.67	7.22 ± 1.00	13.05 ± 2.02	30.55 ± 1.98
HIFc	2.12 ± 0.85	3.35 ± 0.80	6.55 ± 1.70	11.60 ± 2.14	24.00 ± 2.01

[a]Values are expressed as mean ± S.D.; [b]n = 3.

increase bone diseases like arthritis, osteoarthritis, autoimmune diseases [26]. It is found that CFA-induced arthritic animals showed a significant increase in the levels of serum markers whereas treatment with homoisoflavonoid showed a significant decreased in marker enzymes, which indicate potential anti-arthritic activity of homoisoflavonoid. As a consequence of arthritis induced by CFA, the levels of liver marker enzyme *i.e.* ALP, SGOT and SGPT were significant increases ($p < 0.05$) in CFA treated group in comparison to control group. A significant decrease ($p < 0.05$) in these marker enzyme levels were observed in compounds **HIFa1** and **HIFb2** treated a group compared to CFA treated group (Table-2).

TABLE-2
EFFECT OF HIF ON DIFFERENT LIVER MARKER ENZYMES

Treatment groups	ALP (IU/L)	SGOT (IU/L)	SGPT (IU/L)
Control	98.8 ± 5.31	63.5 ± 3.21[b]	69.5 ± 3.15[b]
CFA	161.1 ± 6.95[*]	113.0 ± 5.35[*]	110.6 ± 5.50[*]
Standard	125.5 ± 5.13[*]	76.2 ± 4.21[*]	79.3 ± 4.12
HIFa1	145.5 ± 3.13[*]	95.5 ± 3.41[*]	93.5 ± 4.10[*]
HIFa2	132.0 ± 5.40[*]	83.1 ± 4.14[*]	85.7 ± 5.48[*]
HIFb1	154.2 ± 5.03	110.5 ± 4.50	107.5 ± 4.11
HIFb2	147.7 ± 5.22[*]	91.5 ± 4.15[*]	93.5 ± 4.37[*]
HIFc1	157.3 ± 4.30	109.5 ± 5.01	108.5 ± 5.11
HIFc2	143.1 ± 5.73[*]	93.5 ± 4.27[*]	95.0 ± 3.49[*]

Unit- IU/L, Values are expressed as mean ± SD, n = 6, [*] is $p < 0.05$ is considered as significant, comparison was made as (control group *vs.* CFA treated group), (CFA treated group CFA + std.), (CFA treated group *vs.* CFA + **HIFa1**), (CFA treated group *vs.* CFA + **HIFa2**), (CFA treated group *vs.* CFA + **HIFb1**), (CFA treated group *vs.* CFA + **HIFb2**), (CFA treated group *vs.* CFA + **HIFc1**), (CFA treated group *vs.* CFA + **HIFc2**).

Effect of homoisoflavonoid on *in vitro* anti-inflammatory activity-percent inhibition of protein denaturation: Inhibition of protein denaturation is a common parameter investigated to evaluate the *in vitro* anti-inflammatory activity. The biological function of many proteins gets changed when they undergo the denaturation process one of the important causes for the same is inflammation [27]. Index of inhibition of protein denaturation indicates the significant antiarthritic activity of test compound. Compound **HIFa** showed maximum inhibition of protein denaturation as compared to other derivatives. *In vitro* percent inhibition of protein denaturation is one of the significant methods to screen the anti-inflammatory activity. *In vitro* anti-inflammatory activity of homoisoflavonoid was performed for various concentrations *i.e.* 50, 100, 250, 500 and 1000 µg/mL. Compound **HIFa** showed greater inhibition of protein denaturation as compared to compounds **HIFb** and **HIFc** (Table-3).

Effect of homoisoflavonoid on the DPPH scavenging activity: DPPH is a commonly used method for the investigation of the free radical scavenging activity of test compounds. The effect of homoisoflavonoids on DPPH radical scavenging activity was carried out for the concentration of 50, 100, 200, 400 and 500 µg/mL. The standard drug ascorbic acid was used in the same concentration for the comparison. The data represents concentration dependent DPPH scavenging activity **HIFa**, **HIFb** and **HIFc** at 50, 100, 200, 400 and 500 µg/mL, respectively. The result obtained for compound **HIFa2** showed maximum free radical scavenging activity as compared to compounds **HIFb** and **HIFc** (Fig. 2). Compound **HIFa** showed significant a free radical scavenging activity as compared to other derivatives and its antioxidant potential may be responsible for its antiarthritic and anti-inflammatory activities [28].

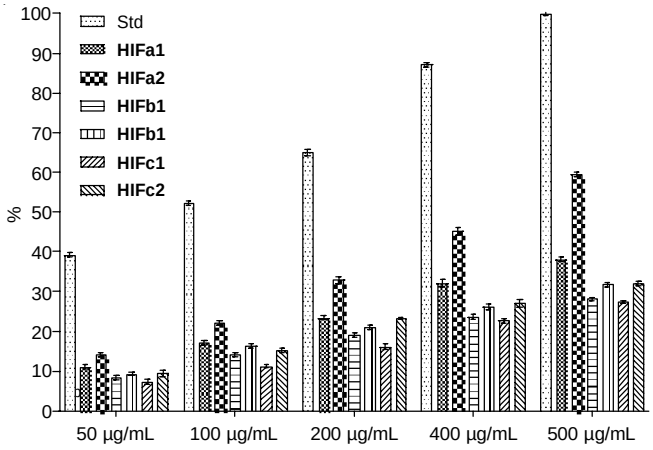


Fig. 2. Effect of HIF's on %DPPH radical scavenging activities; Values are expressed as mean ± SD, n = 3; %DPPH scavenging was plotted against concentration

Conclusion

From the current investigation, it can be concluded that the homoisoflavonoid derivatives showed a significant anti-arthritic, anti-inflammatory and free radical scavenging activities. Amongst them iodo homoisoflavonoid derivatives have greater potential as compare to fluro and hydroxy derivatives.

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TABLE-3
PERCENTAGE INHIBITION OF PROTEIN DENATURATION

Treatment groups	50 µg/mL	100 µg/mL	200 µg/mL	500 µg/mL	1000 µg/mL
Standard	19.02 ± 2.15	32.02 ± 3.09	56.90 ± 3.71	69.62 ± 3.54	88.20 ± 2.95
HIFa	10.11 ± 1.11	17.12 ± 2.05	33.15 ± 3.00	47.11 ± 3.05	53.09 ± 4.50
HIFb	7.56 ± 1.54	12.12 ± 1.84	21.90 ± 2.70	32.02 ± 3.01	43.00 ± 3.77
HIFc	4.92 ± 0.77	9.10 ± 2.85	19.02 ± 3.12	30.54 ± 2.05	40.89 ± 2.61

Values are expressed as mean ± SD, n = 3; IC_{50} for **HIFa**, **HIFb**, **HIFc** was found 941.8, 1162.8 and 1222.0 µg/mL, respectively.

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