*Journal of Enzyme Inhibition and Medicinal Chemistry*, June 2008; 23(3): 341–346

informa
healthcare

# Microbial and xanthine dehydrogenase inhibitory activity of some flavones

C. N. KHOBRAGADE[1], RAGINI G. BODADE[1], M. S. SHINDE[1], DEEPA R. JAJU[1], R. B. BHOSLE[2], & B. S. DAWANE[2]

[1]*School of Life Sciences, Biochemistry Research Laboratory, Swami Ramanand Teerth Marathawada University, Nanded-431606, India, and* [2]*Department of Chemistry, Organic Chemistry Research Laboratory, Yeshwant Mahavidyalaya, Nanded-431 606, India*

(*Received 28 April 2007; accepted 12 June 2007*)

**Abstract**

Xanthine dehydrogenase (XDH) is responsible for the pathological condition called Gout. In the present study different flavones synthesized from chalcone were evaluated in vitro for their inhibitory activity. Inhibitory activity of flavones on XDH was determined in terms of inhibition of uric acid synthesis from Xanthine. The enzymatic activity was found maximum at pH 7.5 and temperature 40°C. The flavones 6-chloro-2-[3-(4–hydroxy-phenyl)-1-phenyl-1-H-pyrazol-4-yl]-chromen-4-one ($F_1$) and 6-chloro-7methyl-2-[3-(4-chloro-phenyl)-1-phenyl-1-H-pyrazol-4-yl]-chromen-4-one($F_2$),were noncompetitive and competitive inhibitor with Ki values 1.1 and 0.22 respectively. The flavones ($F_1$), ($F_2$), 6-chloro-2-[3-(4-chloro-phenyl)-1phenyl-1-H-pyrazol-4-yl]-chromen-4-one($F_3$), 8-bromo-6-chloro-2-[3-(4-chloro-phenyl)-1-phenyl-1-H-pyrazol-4-yl]-chromen-4-one ($F_4$), 2-[3-(4-hydroxy-phenyl)-1-phenyl-1-H-pyrazol-4-yl]-chromen-4-one ($F_5$) and 6-methyl-2-[3-(4-hydroxy-phenyl)-1-phenyl-1-H-pyrazol-4-yl]-chromen-4-one ($F_6$) were also screened for their antimicrobial activity, measured in terms of zone of inhibition. A broad spectrum antifungal activity was obtained against *Trichoderma viridae, Candida albicans, Microsporum cannis, Penicillium chrysogenum* and *Fusarium moniliformae*. In case of *Aspergillus niger* and *Aspergillus flavous* only spore formation was affected, while antibacterial activity was observed against *Staphylococcus aureus, Bacillus subtilis* and *Serratia marsecens* only. The flavones were further analyzed for quantitative structural activity relationship study (QSAR) by using PASS, online software to determine their Pa value. Toxicity and drug relevant properties were revealed by PALLAS software in terms of their molecular weight. Log P values were also studied. The result showed both the $F_1$ and $F_2$ flavones as antigout and therefore supports the development of novel drugs for the treatment of gout.

**Keywords:** *Flavones, inhibitory activity, Xanthine dehydrogenase (XDH), Antimicrobial activity*

## Introduction

Xanthine oxidoreductase (XOR) is a complex metalloflavoprotein which catalyses the conversion of hypoxanthine to xanthine and xanthine to uric acid during protein scavenging pathway with concomitant production of hydrogen peroxides and superoxide anions as the side products. It exhibits two alternate forms of a same gene product as xanthine oxidase (E.C. 1.1.3.22) and Xanthine dehydrogenase (E.C.1.1.1.204) differing in their specifities towards oxygen and $NAD^+$ respectively [1,2] Xanthine oxidoreductase is distributed widely in bacteria, plants and in mammalian tissues mainly in placenta, liver and kidney [3,4].

A large amount of Xanthine oxidase reactivity is observed in milk fat globule membrane of cow, sheep, bovine and human, thereby acting as antimicrobial agent [5,6,7,8]. It has also antitumor activity [9]. Industrially Xanthine oxidase is used to monitor fish freshness in fish industries [10]. An increased activity of xanthine oxidase causes excessive production of uric acid therefore leads to hyperuricemia and renal stone formation. Gout is a systemic disease characterized by deposition of monosodium urate crystal in joint synovia with painful inflammation. The superoxide

Correspondence: Tel: 91(2462)229242. Fax: 91(2462)229245. E-mail: cnkhobragade@rediffmail.com

ISSN 1475-6366 print/ISSN 1475-6374 online © 2008 Informa UK Ltd.
DOI: 10.1080/14756360701608585

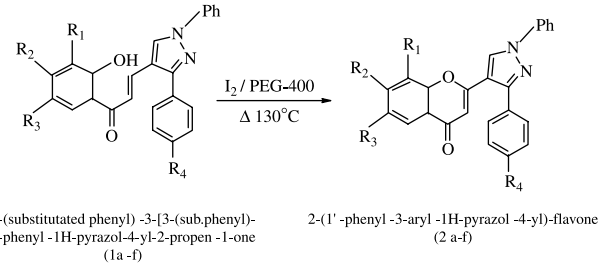
anions produced due to reduction of oxygen causes oxidative injury to tissues by ischemia reperfusion [11,12,13]. Therefore to treat this enzyme induced disorders a number of compounds were screened to inhibit its activity.

Allopurinol is a potent suicide inhibitor of XOR coordinates irreversibly with the reduced form of enzyme thereby inhibits the formation of uric acid [14]. Administration of allopurinol produces several life threatening side effects such as hepatitis, nephropathy and allergic reactions. Apart from these, other inhibitors like 8-bromoxanthine, pteridines, thiazoles, phenyl pyrazoles, aryl triazoles and flavonoids are reported to be as inhibitors of XOR [15,16,17]. Flavonoids of plant origin bears antioxidant activities as well as inhibitory activities against phosphodiesterase, $Ca^{+}$ATPase, aldose reductase, lipooxigenase, cyclooxygenase and Xanthine oxidase [18,19]. Some plant flavonoids of family Celastraceae, Lamiaceae, Polygonaceae, Asteraceae and Lauraceae showed inhibitory activity against XOR [20,21,22]. Bioactive compound of lichen species was also found effective against XO inhibition [23]. Antimicrobial and antituberculosis activities of flavonoids are also reported [24,25,26]. Therefore in the present work we report the synthesis of derivatives of flavones from chalcone with reduced side effects and increased therapeutic values. The catalytic behavior of chicken XDH is examined on xanthine with $NAD^{+}$ as electron acceptor at variable pH, temperature, and substrate and inhibitor concentration on XDH. Furthermore the antimicrobial activity of all the flavones (F1 to F6) is also studied against some pathogenic bacteria and fungi. Supplementary to this work the quantitative structural activity relationship (QSAR) is analyzed *In silico* for two derivatives to predict their pharmacological activities by using PASS online software for Pa values. The toxicity and drug relevant properties of flavones were studied using Osiris and PALLAS software.

## Materials and methods

### *Materials*

Xanthine, uric acid, $NAD^{+}$, nystatin and tetracycline were purchased from HiMedia Laboratories Ltd. Mumbai, India. All chemicals were of AR grade and used without further purification unless stated otherwise. Pathological strains of bacteria and fungi were procured from Institute of Microbial technology (IMTech) Chandigarh, India and maintained on Nutrient agar under aerobic condition for 24 h at 37°C and fungal cultures on Potato dextrose agar for 24–48 h at 30°C [27]. The chicken liver was collected from the local market and preserved at 10°C.




1-(substitutated phenyl) -3-[3-(sub.phenyl)-1-phenyl -1H-pyrazol-4-yl-2-propen -1-one (1a -f)

2-(1' -phenyl -3-aryl -1H-pyrazol -4-yl)-flavone (2 a-f)

Figure 1. Synthesis of flavones.

### *Synthesis of flavones*

Chalcones were prepared by base catalyzed condensation of appropriately substituted ketones with substituted aldehydes [28]. Flavones were synthesized by dissolving 2′-Hydroxy substituted chalcone in 15 mL of poly ethylene glycol (PEG-400) and treated with catalytic amount of iodine. Whole contents were heated at 140°C for 3 h in oil bath. After overnight incubation the product was washed with cold water, followed by 10% sodium thiosulfate solution for several times. The precipitated flavone was collected and recrystallised from methanol to yield pure flavone. This typical experimental procedure was used to prepare the other analogues of this series (Figure 1). Thin layer (TLC) and melting point (M P) of all the flavones were checked to confirm their purity. IR and NMR data was analyzed by FTIR (Perkin Elmer model RXI spectrophotometer) and AVANCE 300 MHz spectrophotometer in DMSO-$d_6$ using TMS as an internal standard respectively (Table–I).

### *Extraction and purification of enzyme*

The Xanthine dehydrogenase was extracted by standard method from fresh chicken liver [3]. It is homogenized with five volumes of distilled water for 3 min. The homogenate was transfered to a boiling water bath and brought to 70°C with mechanical stirring. After 2 min the temperature was reduced to 55°C by transferring the homogenate to an ice bath. The extract was centrifuged at 9000 rpm for 20 min in high speed centrifuge (Suprspin RNV-Plasto craft).The clear supernatant solution was cooled

Table I. Structure of flavone derivatives.

| | Substitutents | | | | | |
|---|---|---|---|---|---|---|
| Flavone | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Yield(%) | M.P. (°C) |
| $F_1$ | H | H | Cl | OH | 85 | 210 |
| $F_2$ | H | $CH_3$ | Cl | Cl | 85 | 195 |
| $F_3$ | H | H | Cl | Cl | 80 | 212 |
| $F_4$ | Br | H | Cl | Cl | 80 | 182 |
| $F_5$ | H | H | H | OH | 90 | 230 |
| $F_6$ | H | H | $CH_3$ | OH | 85 | 196 |

in an ice bath and enzyme was precipitated by addition of ammonium sulphate (70%) with continuous stirring. The precipitate was collected by recentrifugation at 9000 rpm for 15 min. The precipitate was dissolved in 6–7 volume of 0.05 M potassium phosphate buffer pH 7.9 containing 0.1 mM ethylenediaminetetra acetic acid (EDTA). The purification was further carried out by 38% v/v and then 47% v/v cold acetone. The precipitate was separated by centrifugation and dissolved in 0.1 mM EDTA containing deionised water. The final step of purification was accomplished by dialysis against deionised water for 38 h at 4°C. The dialyze itself was used immediately as crude enzyme for measurement of enzyme activity and protein estimation.

*Protein measurement and enzyme assay*

The protein content of the enzyme was determined from the standard curve of BSA by Folin Lowry method [29]. One unit of enzyme activity was defined as the amount of enzyme responsible for production of 1 μmol of uric acid in 1 mL of assay volume per min at 25°C and pH 7.5. The enzyme activity was measured by a standared method [30]. The reaction mixture contains 1.9 mL of 50 mM potassium phosphate buffer of pH 7.5, 1 mL of 0.15 mM Xanthine as substrate, 0.1 mL of native XDH (0.22U/mL) and 0.5 mL of 0.5 mM of $NAD^+$ as reducing agent, all the contents were mixed by swirling and incubating at $37^0$C for 5 min. An increase in absorbance at 293 nm (UV–Vis Shimadzu) after the enzyme treatment confirms the production of uric acid from substrate. The formed uric acid concentration was estimated by standard curve of uric acid [31]. The blank was prepared without enzyme solution.

*Kinetic assay*

Assays were performed by using the better physiological condition for optimization of enzyme activity at variable pH ranging from 2 to 9 and temperature from 10°C to 80°C. The phosphate buffer of varying pH was selected for pH effect study only. Effect of different substrate concentration (1 μg/mL to 6 μg/mL) was studied for determining the stability of enzyme substrate complex in terms of kinetic parameters $K_m$ and $V_{max}$ values. The optimum pH, temperature and substrate concentration was examined by the same procedure used for enzyme activity study.

*Enzyme inhibitory activity*

Enzyme inhibitory activity was determined by quantifying the amount of uric acid produced from xanthine in the reaction mixture. Inhibition of XDH catalyzed reaction by flavones as inhibitor was measured by observing the decrease in the uric acid formation at 293 nm. The assay mixture contains both flavone and xanthine in order to have equal competition of the substrate and inhibitor for enzyme active site. Both the inhibitor and substrate concentrations were maintained identical.

*Antimicrobial activity*

The antimicrobial activity of flavones was evaluated by agar diffusion method [32,33]. Stock solution of flavones were diluted in dimethyl sulphoxide (DMSO) to give dilutions ranging from 10 μg/ml to 250 μg/mL used for antimicrobial assay. For antifungal activity different fungal spore suspension in sterile distilled water were adjusted to give a final concentration of $10^6$ cfu/mL. An inoculum of 0.1 mL spore suspension of each fungus was spreaded on Sabourauds Dextrose agar plates. While for antibacterial activity nutrient agar was used. It is seeded with 0.1 mL of respective bacterial culture strains suspension prepared in sterile 0.85% saline of $10^5$ cfu/mL dilution. The wells of 6 mm diameter were filled with 0.1 mL of each flavone dilutions separately for each test fungi and bacterial strain. The DMSO alone was used as control. The nystatin 30 μg/mL and tetracycline 10 μg/mL was used as reference antifungal and antibacterial substance respectively for comparison. Inoculated plates were incubated for 24 h at 37°C ± 0.5°C for antibacterial activity and 48 h at 28 ± 0.2°C for antifungal activity. The antimicrobial activity was measured in terms of the zone of inhibition in mm. Minimum inhibitory concentration (MIC) was determined as the lowest compound concentration which completely inhibit the fungal and bacterial growth after incubation time (Table II).

*Drug prediction analysis*

All the computational work was performed using the BioMed Cache, a computer aided molecular design modeling tool developed for Microsoft operating system. Pharmacological activities of flavones were developed by QSAR study using PASS (Prediction of activity spectrum for substance) an online software. It is used to determine Pa values. Osiris property explorer an organic chemistry portal was used for evaluation of toxicity and drug relevant properties of flavones. While PALLAS 3.1.1.2 a computer tool was used for studying molecular weight, hydrogen bond donor count (HBD), and hydrogen bond acceptor (HBA). Log P value was studied according to Lipinski rule of 5 in the form of Lipinski score.

## Results

The synthesized flavone derivatives of chalcone were characterized by IR and NMR spectral analysis. The compound $F_1$ shows prominent peaks in IR:

Table II. Antimicobial activity of flavones in DMSO solvent (Inhibition zone in mm).

| | Bacteria | | | | | | | | Fungi | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Flavones | EC | ST | PV | PA | SA | BS | BM | SM | AN | TV | PC | AF | MC | CA | FM |
| F1 | ± | − | ± | ± | 15 | 10 | − | 12 | ++ | 25 | 11 | − + | ± | 15 | 15 |
| F2 | ± | − | ± | ± | 10 | 11 | − | 9 | ++ | 27 | 9 | ++ | 15 | 13 | 25 |
| F3 | ± | − | ± | − | 12 | 20 | − | ± | ++ | 23 | 11 | ++ | 10 | − | 15 |
| F4 | ± | − | ± | ± | 15 | 15 | − | 15 | ++ | 20 | 13 | ++ | 16 | 20 | − |
| F5 | ± | − | ± | − | 11 | 10 | − | ± | − + | 22 | 9 | ++ | 10 | − | − |
| F6 | − | − | 6 | − | ± | ± | − | − | ++ | 16 | 15 | ++ | − | ± | 10 |
| Control | ± | − | ± | ± | 10 | 11 | − | 10 | − + | 25 | ± | ++ | ± | ± | 30 |
| Tetracycline | − | 32 | 25 | 34 | 33 | 29 | 27 | 20 | − | − | − | − | − | − | − |
| Nystatin | − | − | − | − | − | − | − | − | 14 | 18 | 17 | 14 | 8 | 17 | 10 |

Data represent the mean of three eplicates.
EC-*Escherichia coli*; ST-*Salmonella typhii*; PV-*Proteus vulgaris*; PA-*Psudomonasaeruginosa*; SA-*Staphylococcus aureus*; BS-*Bacillus substilis*; BM-*Bacillus megaterium*; SM-*Serraia marcescens*; FM-*Fusarium monihformae*; AN-*Aspergillus niger*; PC-*Penicillium chrysogenum*; TV-*Trichoderma viridae*; CA-*Candida albicans*; MC-*Microsporum cannis*.
Not detected − ; Trace activity ± ; More spore area affected++; Less spore area affected −+.

λmax spectra at 3425.3,3068,1782,1664,1596, 1285 $cm^{-1}$, $F_2$ 3070.5,1660.5,1598.9,1305.7 $cm^{-1}$ , $F_3$ at 3069,1662,1601,1302 $cm^{-1}$, $F_4$ at 3070.5, 1664.5,1598.9,1310 $cm^{-1}$, $F_5$ 3340,3035.5,1662.0, 1601,1300 $cm^{-1}$ and $F_6$ 3320,3025.8,1660.2, 1599.8,1305 $cm^{-1}$.While NMR data obtained at $F_1$ $^1$H-NMR (DMSO-$d_6$): δ = 7–8.20(m,12H,Ar-H),8.80(s, 1H, H-5 of pyrazole) 11.30(s,1H,Ar-OH) 6.60(s,1H,H-3 of pyrone), $F_2$ $^1$H-NMR (DMSO-$d_6$): δ = 2.40(s,3H,Ar-$CH_3$)6.50(s,1H, H-3 of pyrone) 7.20–8.10(m,11H,Ar-H) 9.13(s,1H,H-5 of pyrazole), $F_3$ $^1$H-NMR (DMSO-$d_6$): δ = 7.01–8.24(m,12H,Ar-H) 9.05(s, 1H,H-5 of pyrazole) 6.01(s, 1H, H-3 of pyrone) $F_4$ $^1$H-NMR (DMSO-$d_6$): δ = 7.04–8.30(m, 11H,Ar-H)9.20(s,1H,H-5 of pyrazole) 6.58(s, 1H,H-3 of pyrone), $F_5$ $^1$H-NMR (DMSO-$d_6$): δ = 10.8(s,1H, 0H) 6.98–7.10(m,13H,Ar-H) 6.62(s,1H,H-3 of pyrone) 9.20(s,1H,H-5 of pyrazole), $F_6$ $^1$H-NMR (DMSO-$d_6$): δ = 2.15(s,3H,Ar-$CH_3$)6.99–7.08(m, 12H,Ar-H) 6.58(s, 1H,H-3 of prone) 11.02(s, 1H, OH) 9.08(s, 1H,H-5 of pyrazole).

Enzyme activity of the dialyzed enzyme was found to be 0.22 U/mg and used for further kinetic study of enzyme. The pH profiles for enzyme activity at variable ranges revealed an optimum at pH 7.5. The pH above and bellow to this value causes denaturation of enzyme. The temperature effect was studied at variable temperatures and showed optimum activity at 40°C. The substrate concentration effect on enzyme was studied at variable concentration from 1 to 6 μg/mL. Maximum enzyme activity was obtained at 5 μg/mL where enzyme get completely saturated with Xanthine. The values of $K_m$ 2.5 μg/mL and $V_{max}$ 0.1 mg/min were obtained by using Lineweaver-Burk plot [35]. Inhibitory effect of the two flavones (F1 and F2) on the uric acid formation from substrate was studied at variable inhibitor concentration (2, 3, 5 μg/mL) against variable concentration of Xanthine (1–5 μg/mL). In case of flavones, compound F1 found to be non competitive inhibitor with $K_i$ 1.1. The $K_m$ values were constant while $V_{max}$ consequently decreased with increased inhibitor concentration (Figure 2). Compound F2 revealed increase in the $K_m$ values while $V_{max}$ remained constant at all values of inhibitor concentrations, thus confirmed the competitive type of inhibition. The Lineweaver-Burk [15] equation was used for the calculation of $K_i$ value and it was obtained as 0.22 (Figure 3).

The flavones were also evaluated for antimicrobial activity against some pathogenic fungi and bacteria. The result showed variable toxicity of flavones against different fungi and bacteria. This may be attributed to the different structures and functional groups attached to the basic nucleus of Chalcone. The sensitivity pattern of fungi revealed *Trichoderma viridae* > *Penicillium chrysogenum* > *Fusarium moniliformae* > *Micromonosporum cannis* against the majority of flavones, while *Candida albicans* found to be quite resistant. *Aspergillus niger* and *Aspergillus flavous* showed spore inhibition pattern where all black and

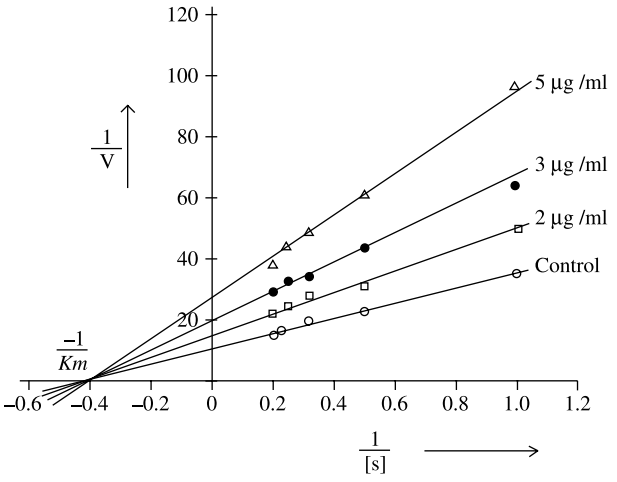



Figure 2. Lineweaver-Burk plot of inhibition of XDH by flavone $F_1$ with xanthine(1-5 μg/mL), enzyme (0.22 U/mg) in potassium phosphate buffer (50 mM, pH 7.5) to a total volume of 3.05 mL.

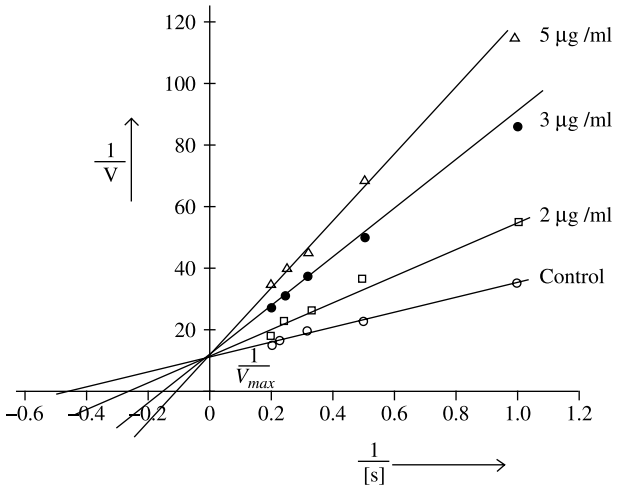



Figure 3. Lineweaver-Burk plot of inhibition of XDH by flavone F2 with xanthine (1-5 µg/mL), enzyme (0.22 U/mg) in potassium phosphate buffer (50 mM, pH 7.5) to a total volume of 3.05 mL).

green spores were turned in to gray. *Serratia marcesense*, *Bacillus subtilis* and *Staphylococcus aureus* were inhibited maximally by flavones $F_1$, $F_3$ and $F_2$. QSAR study of $F_1$ and $F_2$ provide important physiological properties for XDH inhibition activity. The results suggested that the XDH inhibitory activity was depends on molecular weight and polarity of flavones. $F_1$ with Pa values 0.556 and $F_2$ with 0.505 were found to be nearer to the standard Pa value 0.500. $F_1$ M W (414.840) was effective while $F_2$ M W (447.321) was inefficient than standard M W (500). The Pa and M W study will predict its role in gout treatment. Log p values of $F_1$ (4.658) and $F_2$ (5.927) gives important information about hydrophobicity and hydrophilicity of flavones and found to be effective as values are nearer to standard Log p 5 [36].

## Discussion

The differences in enzymatic activity at optimum pH 7.5 and temperature at 40°C shows deviation from the other enzyme sources like cow and goat milk, this may be due to nature of their sources [7]. Earlier experimental works suggested that chalcone based flavones and falvonoids are found to be effective as competitive and noncompetitive inhibitors for XOR [20–25].The noncompetitive type of inhibition is reversible and can not be overcome by increasing substrate concentration. The inhibitor binds to the enzyme and enzyme substrate complex. The competitive mode of inhibition can be reversed by increasing substrate concentration.The inhibitor binds only to the free enzyme. The $K_m$ values indicate affinity of an enzyme towards substrate, the greater the value of $K_m$ the less is the affinity. It is suggested that chalcone falvonoids and flavones found more active as inhibitor in the chalcone than in the closed ring form and also found to be depending on substituted groups of the chalcone rings. The compound having —OH group at C 3,4,5, 6, 7 and a keto group improves the inhibitory activity [12,16], while —OH at C 2 retards the inhibitory activity. The compound $F_1$ posses both hydroxyl and keto groups while compound $F_2$ contains keto and methyl group [28] therefore confirms inhibitory activity against XDH.

An improved antifungal activity may be due to the halide groups like Br, Cl, F and $N0_2$ affecting particularly the cell wall synthesis. Therefore the flavone $F_4 > F_2 > F_3 > F_1 > F_5 > F_6$ shows maximum antifungal activity and flavone $F_2 > F_3 > F_1 > F_4 > F_5 > F_6$ shows maximum antibacterial activity with $CH_3$, Cl;Cl,Cl;Cl,OH;Br,Cl;OH;$CH_3$, OH groups.This is also supported by the previous reports [34]. The present study therefore is useful to develop highly selective and useful drugs in medical investigation against fungal diseases also, since mammalian cells are devoid of cell wall. *Insilco* studies of flavones conclude that $F_1$ and $F_2$ could be used in designing an inhibitor for XDH against gout.

## References

[1] Ling Li. Free radicals in Biology and Medicine (Spring) 2001; 77(222):1.
[2] Russ Hill, Takeshi Nishino. The FASEB Jour 1995;6:995.
[3] Rajagopalan KV, Philip Handler. J Biol Chem 1967;242(18): 4097.
[4] Many A, Wester hausen - Larson, Kanbour-Shakir A, Roberts JM. Placenta 1996;17(5):361.
[5] Andrew M Ventom, Janet Deistung, Robert C Bray. Bichem J 1988;255:949.
[6] Mustapha Benboubetra, Abderahmene Baghiani, Djebbar Atmani, Roger Harrison. Dairy Sci 2004;87:1580.
[7] Evans C Egwim, Mohammed A Vunchi, Patience, O Egwim. Biokemistri 2005;17(1):1.
[8] Hannah M Martin, John T Hancock, Salisbury V, Roger Harrison. Infect Immun 2004;72(9):4933.
[9] Greed WA, Bell A, Urem JR. Chemistry and Biochemistry. Vol 1. Adorjan Aszalos Inc Florida: CRC Press; 1981. p 147.
[10] Swaisgood NE. Food Enzymology (In Elsevier NewYork) 1991;182.
[11] Oetti K, Reibnegger G, Pteridines. Biochem Biophys Acta 1999;1430:387.
[12] Cos P, Ying L, Calomme M, Hu JP, Cimamga K, Van Poel B, et al. J Nat Prod 1998;1(61):71.
[13] Benjamin LJ, Godber Guenter Schwarz, Ralf R Mendel, David J Lowe, Robert C Bray, Rot Roger Harrison. Bichem J 2005;388(2):501.
[14] Ken Okamoto, Bryan T Eger, Tomoko Nishino, Shiro Kondo, Emil F Pai, Takeshi Nishino. J Biol Chem 2003;278(3):1848.
[15] Hemlata Tamta, Sukirti Kalra Anup, Mukhopadhyay K. Biochemistry 2005;2(BM 05):106.
[16] Beiler JM, Gustav J Martin. J Biol Chem 1951;19:831.
[17] Russ Hille, Richard C Stewart. J Biol Chem 1984;259(3): 1570.
[18] Akihiko Nagao Michikoseki, Hidetaka Kobayashi. Biosci Biotechnol Biochem 1999;63(10):1787.
[19] Toshimitsu Hayashi, Kazuko Sawa, Masaru Kawasaki, Munehisa Arisawa, Mineo Shimzu, Naokata Morita. J Nat Prod 1988;51(2):345.
[20] Mai Thanh Thi Nguyen, Suresh Awale, Yasuhiro Tezuka, Jun-ya Ueda, Quan Le Tran, Shigetoshi Kadota. ISSN Planta Med 2005;32.

[21] Kongh D, Cai Y, Huang WW, Cheng CH, Tan RX. J Ethnophar 2000;73(1-2):199.
[22] Gonzalez AG, Bazzocchi RL, Moujirl Ravelo AG, Correa MD, Gupta MP. J Ethnophar 1995;46(1):25.
[23] Behera BC, Urmila Makhija. Current Sci 2002;82(1)61.
[24] Yuh – Meei Lin, Yasheen Zhou, Michael T Flavin, Li-ming Zhou, Weiguo Nie, Fa Ching Chen. Bioorg and Med Chem 2002;10:2795.
[25] Yoichi Sato Suzaki, Takako Nishikawa, Masaru Kihara, Hirofumi Shibata, Tomihiko Higuti. J Ethnophar 2002; 72(3):483.
[26] Ehsan Ullah Mughal, Muhammad Ayaz, Zakir Hussain, Aurangzeb Hasan, Amina Sadiq, Muhammad Riaz, Abdul Malik, Samreen Hussain, Choudhary M Iqbal. Nat Prod 1999;62(6):909.
[27] Institute of Microbial Tech. MTCC Catalogue of strain Chandigarh, India 2005. p 130.
[28] Simon F Nielsen, Mogens Larsen, Thomas Boesen, Schnning Kristian, Hasse Kromann. J Med Chem 2005;48:2667.
[29] Lowry OH, Rosebrough NJ, Far AL, Randall RJJ. Biol Chem 1951;193:265.
[30] Bergmeyer HU, Gawehn K, Grassl M. 19. New York: Academic Press; p 521.
[31] Chen XB, Gomes MJ. International Feed Resource unit UK. 1992;1.
[32] Gould JC, Bowie JH. J Edinb Med 1952;59:178.
[33] Anna King, Brown DFJ. J Antimicrob Chemother 2001;48:71.
[34] Paula Boeck, Paulo C Leal, Rosendo A Yunges, Valdir Cechinel Filho, Andrea Escalante, Ricardo LE furian, Susana Zacchino. Arch Pharm Chem Life Sci 2005;338:87.
[35] Donald V, Judith VG. In Biochemistry. NewYork: John Wiley - Sons; 2004. p 472–487.
[36] Kamlet M, Abboud JL, Abraham M, Taft R. J Org Chem 1983;48:2877.