Australian Journal of Basic and Applied Sciences, 6(1): 117-122, 2012
ISSN 1991-8178

Purification of Xanthine Oxidase Inhibitor from *Carica papaya* Leaves using Reversed Phase Flash Column Chromatography (RPFCC) - High Performance Thin Layer Chromatography (HPTLC)

Saiful M.N. Azmi, Parveen Jamal and Azura Amid

Bioprocess and Molecular Engineering Research Unit (BPMERU), Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia (IIUM), P.O. BOX 10, 50728 Kuala Lumpur, Malaysia

Abstract: Xanthine oxidase (XO) is an enzyme that catalyses the metabolism of hypoxanthine and xanthine into uric acid. It is responsible for the medical condition known as gout, which is caused by the deposition of uric acid in the joints leading to painful inflammation. Inhibition of XO leads to remission in gout. *Carica papaya* is a member of the small family *Caricaceae* allied to the *Passifloraceae*. In folk medicine, *Carica papaya* has been used as an important traditional herbal medicine due to its vast bioactive compounds found including kaempferol, quercetin, 5, 7-dimethoxycoumarin, alkaloids, carpaine and pseudocarpaine. In this study, the dried mature leaves of *Carica papaya* was extracted with distilled water, optimized to obtain higher XO inhibitory activity, and the extract was subjected to reversed-phase flash column chromatography (RPFCC) and high performance thin layer chromatography (HPTLC) for the purification. Preliminary identification by chemical screening conducted on the optimized sample of distilled water extract of *Carica papaya* leaves showed the presence of several secondary metabolites, mainly, flavonoids, alkaloids, saponins, xanthine alkaloids, terpenoids and anthranol glycosides which could partially explain the pharmacological properties of this plant and demonstrates its importance in alimentation and daily intake especially for gout patient.

Key words: *Carica papaya*; xanthine oxidase inhibitor; gout; reversed-phase flash column chromatography

INTRODUCTION

Xanthine oxidase (XO), a large protein with a molecular weight of 270,000 is an enzyme that catalyzes the oxidation of hypoxanthine and xanthine to uric acid in the purine catabolic pathway. The presence of uric acid which is normally dissolved in the blood may, from time to time, form microscopic crystals in the joint, subsequently leads to severe inflammatory arthritis which is called acute gouty arthritis or acute gout (Janssens *et al*., 2006). Five to thirty percent of the global population suffers from overproduction or underexcretion of uric acid (Umamaheswari *et al*., 2007; Owen and Johns, 1999) which leads to hyperuricemia, an important risk factor for gout (Shimoto *et al*., 2005). The XO is also a major source of free radicals (superoxide) and plays an important role in various ischemic tissues, vascular injuries and inflammatory diseases. Its activity also contributes significantly to oxidative stress, brain edema, respiratory syndrome, viral infection, thermal stress and hemorrhagic shock in vivo (Naoghare *et al*., 2010).

Many components of human diet such as vegetables and fruits are excellent inhibitors of XO (Selloum *et al*., 2001; Cotelle *et al*., 1996) Our preliminary screening study revealed that an aqueous extract of *Carica papaya* mature leaves have promising activity to inhibit XO with 75.68 ± 0.14%. Optimization of process conditions for extraction was conducted to maximize XO inhibitory activity and *Carica papaya* leaves has demonstrated 88.68 ± 0.6% inhibitory activity at a concentration of 100 μg/ml, only 5.01% less than the activity exhibited by allopurinol (93.69 ± 0.2%), a commercial XO inhibitor (XOI), evaluated at the same concentration.

In folk medicine, *Carica papaya* has been used to treat diabetes mellitus and hypertension. Papaya leaves contain the bitter alkaloids, carpaine and pseudocarpaine, which act on the heart and respiration (Perry and Metzger, 1980). Dried leaves have been smoked to relieve asthma or as a tobacco substitute. The extracts of ripe and unripe papaya fruits as well as the seeds are active against gram-positive bacteria. Strong doses are effective against gram-negative bacteria. The seeds are anti-inflammatory, analgesic, and also used to treat stomach ache and fungal infections. The mature (ripe) fruit treats ringworm whereas the green fruits treat high blood pressure, and are used as an aphrodisiac (Singh *et al*., 1980).

Chemical screening was conducted qualitatively and quantitatively as preliminary identification of the active secondary metabolites that may have contributed to the stronger XO inhibition. The potential bioactive compound could be used subsequently as anti-gout supplement. Reversed-phase flash column chromatography

Corresponding Author: Parveen Jamal, Bioprocess and Molecular Engineering Research Unit, Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia (IIUM), P.O. BOX 10, 50728 Kuala Lumpur, Malaysia
E-mail: jparveen@iium.edu.my

(RPFCC) and high performance thin layer chromatography (HPTLC) were employed for the purification of the XOI compound as their combination is able to greatly increase the purity of the isolated compounds and the productivity of the process while allowing more diversity in the samples to be routinely evaluated (Bickler, J. R., 2001). There is an urgent need and trend to develop new XOI from natural source (Umamaheswari *et al*., 2007) as inhibition of XO is also an important strategy in the formulation of preservation media for organ transplantation purposes (McCord and Fridovich, 1968). In addition, inhibition of XO has also been suggested for the treatment of hepatitis and brain tumor because increased serum XO levels are found in hepatitis and hepatotoxicity as well as in brain tumors (Kokoglu *et al*., 1990).

MATERIALS AND METHODS

Chemicals and Reagents:

Allopurinol, xanthine and xanthine oxidase (buttermilk) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Dimethylsulphoxide (DMSO), *p*-anisaldehyde reagent, molybdatophosphoric acid spray solution, ninhydrin spray solution, dragendorff's reagent spray solution (contain acetic acid), methanol (MeOH), absolute ethanol (EtOH), dichloromethane (CH_2Cl_2), ethyl acetate (EtAc), diethyl ether (DiEt) and other chemicals or reagents of analytical grade were obtained from Merck (Darmstadt, FR Germany). Potassium di-hydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) were of the highest purity.

Plant Materials:

The leaves of *Carica papaya* were collected fresh from its natural habitat in the state of Selangor, Malaysia on July, 2009. The specimen was authenticated by the Department of Biotechnology Engineering, IIUM, Malaysia.

Preparation of the Extract:

The leaves were soaked in water, washed to get rid of any adhering dust and impurities, and then oven dried at 40°C for 72 hours. The dried leaves were ground into powdered-like particles and stored in a –20°C freezer prior to extraction process. Distilled water was added to the ground leaves triturate to 20:1 (v/w), capped with aluminum foil, and placed in a shaker incubator. The agitation speed of the shaker incubator was set at 125 rpm and ran for 15 hours at 30°C. The mixture of plant material and extraction solvent was filtered using Whatman No. 1 filter paper and the filtrate was collected, concentrated by vacuum rotary evaporator and dissolved in DMSO (100%), subjected to the XO inhibitory activity assay spectrophotometrically at 295 nm.

Xanthine Oxidase Inhibitory Activity of Allopurinol:

Allopurinol (synthetic XOI) was prepared in various concentrations (100, 75, 50, 25, 10, 5, 0.5, 0.1, 0.05 µg/ml) to determine the highest capacity of its inhibitory activity, subsequently regarded as the standard concentration for the analysis.

Xanthine Oxidase Inhibitory Activity Assay:

The inhibitory effect on XO was measured spectrophotometrically at 295 nm under aerobic condition following the existing method with some modifications (Umamaheswari *et al*., 2007; Unno *et al*., 2004). Allopurinol at a concentration of 100 µg/ml was used as a positive control for the inhibition test. The reaction mixture consisted of 300 µl of 50 mM sodium phosphate buffer (pH 7.5), 100 µl of sample solution dissolved in distilled water or dimethylsulphoxide (DMSO), 100 µl of freshly prepared enzyme solution (0.2 units/ml of xanthine oxidase in phosphate buffer) and 100 µl of distilled water. The assay mixture was pre-incubated at 37°C for 15 minutes. Then, 200 µl of substrate solution (0.15 mM of xanthine) was added into the mixture. The mixture was incubated at 37°C for 30 minutes. Next, the reaction was stopped with the addition of 200 µl of 0.5 M hydrochloric acid. The absorbance was measured using UV/VIS spectrophotometer against a blank prepared in the same way but the enzyme solution was replaced with the phosphate buffer. Another reaction mixture was prepared (control) having 100 µl of DMSO instead of test compounds in order to have maximum uric acid formation.

The degree of XO inhibitory activity was calculated by using Eq. (1), where α is the activity of XO without test extract and β is the activity of XO with test extract (Naseem *et al*., 2006).

$$\% \text{ XO inhibition} = (1 - \beta/\alpha) \times 100 \quad (1)$$

Qualitative and Quantitative Analysis of the Secondary Metabolites:

The optimized crude extract of *Carica papaya* leaves was subjected to chemical screening for preliminary identification of the secondary metabolites that may be accounted for XO inhibitory activity employing previously described methodologies (Bhat *et al*., 2008; Harborne, 1984; Marini-Bettolo *et al*., 1981; Harborne,

1973; Fransworth, 1966). The secondary metabolites detected in the optimized crude extract of *Carica papaya* leaves were quantitatively determined according to the previously reported methodologies (Bohm and Koupai-Abyazani, 1994; Edeoga *et al*., 2005; Obadoni and Ochuko, 2001; Harborne, 1998).

Isolation and Purification of the XOI Compound:

Preparative TLC was used as a preliminary technique for the isolation of the active compounds in the optimized extract of *Carica papaya* leaves. Pre-coated TLC plates of silica gel 60 F_{254} (Merck) (2.5 cm X 10 cm) were used for preparative TLC. Eighteen mobile phases were used (M1: dH_2O-Hexane; M2: MeOH-CH_2Cl_2; M3: EtOH-CH_2Cl_2; M4: Acetone-CH_2Cl_2; M5: EtAc-CH_2Cl_2; M6: DiEt-CH_2Cl_2; M7: dH_2O-Hexane; M8: MeOH-Hexane; M9: EtOH-Hexane; M10: Acetone-Hexane; M11: EtAc-Hexane; M12: DiEt-Hexane; M13: dH_2O-EtAc; M14: MeOH-EtAc; M15: EtOH-EtAc; M16: Acetone-EtAc; M17: CH_2Cl_2-Hexane; M18: MeOH-EtOH), each with decreasing and then, increasing stepwise polarity (9:1, 8:2, 7:3, 1:1, 3:7, 2:8, 1:9). Zones were detected under UV (at 254 and 366 nm) and in daylight after spraying with acidic *p*-anisaldehyde reagent (heating at 105°C for 5 min), molybdatophosphoric acid spray solution, ninhydrin spray solution, dragendorff's reagent spray solution (contain acetic acid), and fuming with ammonia vapour. Then, the optimized extract was subjected to RPFCC using a 20 g ISOLUTE C_{18} pre-packed disposable column (Argonaut Technologies' FlashMaster™), eluted with the best solvent systems selected from the preparative TLC separately (50 ml each), with each flow rate was fixed at 5 ml/min. Each liquid fraction obtained from RPFCC was further analyzed using HPTLC silica gel 60 F_{254} (Merck) as their combination is able to greatly increase the purity of the compound. The single spot appeared on the HPTLC was compared with the separated constituents on the preparative TLC based on their retardation factor (R_f).

RESULTS AND DISCUSSION

Carica papaya is an ancient herbal medicinal plant, which is rich in its medicinal value. Many bioactive compounds contained in papaya at higher level have been reported to play a vital role in biochemical/enzymatic processes and are likely to be responsible for its medicinal properties such as wound healing.

The capacity of distilled water to dissolve many of the bio-molecules found in living organisms made it an exceptional medium for metabolic reactions. Biochemical substances that are ionic as well as those that are polar or uncharged are soluble in water. Many uncharged bio-molecules readily dissolve in water because they have polar functional groups that form favorable dipole-dipole interactions (Boyer, R., 1998). A few examples of these compounds are alcohols, amines, amides, and esters; which may have contributed to the inhibitory activity of xanthine oxidase in *Carica papaya* leaves as leaves of a plant are well recognized to contain major secondary metabolites credited for various biological activities.

Much plant analysis is devoted to the isolation and identification of secondary constituents in a particular species or group of species. Therefore, it is essential to monitor the extraction and separation procedures at each stage in order to follow the active material as it is purified. The activity can sometimes vanish during fractionation (Harborne, 1998). The choice of chromatographic techniques for separation depends largely on the solubility properties and volatilities of the compounds to be separated. In the present attempt to isolate XOI from *Carica papaya* leaves, TLC was employed due to several reasons which include versatility, speed and sensitivity. Versatility is due to the fact that a number of different adsorbents (i.e. silica gel, aluminum oxide, polyamide, Sephadex, magnesium phosphate and ion exchange resin) may be spread on to a glass plate or other support and employed for chromatography. The greater speed of TLC is due to the more compact nature of the adsorbent when spread on a plate and is an advantage when working with labile compounds. Finally, the sensitivity of TLC is such that separations on less than μg amounts of material can be achieved if necessary [24]. Flash column chromatography has become one of the most commonly used preparative separation and purification techniques in organic synthesis, drug discovery, pharmaceutical intermediate purification and many other applications. HPTLC, which is a TLC plate coated with the same fine microparticles of silica that are used in the columns for HPLC usually gives more efficient and rapid separations than on conventional silica layers.

Xanthine Oxidase Inhibitory Activity of Allopurinol:

Allopurinol has exhibited the highest XO inhibitory activity at a concentration of 100 μg/ml (93.69 ± 0.2%) as represented in Table 1.

Identification of the Active Compounds that may Responsible for XO Inhibitory Activity:

Chemical screening conducted on the optimized sample of distilled water extract of *Carica papaya* leaves showed the presence of several secondary metabolites, mainly, phenolic compounds, flavonoids, alkaloids, tannins, saponins and xanthine alkaloids, anthracene-derivative glycosides and terpenoids (Fig. 1). Any of these metabolites may have contributed to the stronger XO inhibition. Many flavonoids including myricetin, apigenin, quercetin and isovitexin have been reported to inhibit XO (Ponce *et al*., 2000; Lin *et al*., 2002). A study reported

that a hydroxyl moiety at C7 and C5 of the apigenin and the carbonyl group at C4 of the XO contribute favourable hydrogen bonds and electrostatic interactions between inhibitors and the active site (Lin *et al*., 2002).

Table 1: XO Inhibitory Activity of Allopurinol at Various Concentrations

Concentration (µg/ml)	XO inhibitory activity (%)
100	93.69 ± 0.2
75	89.37 ± 0.5
50	81.54 ± 3.07
25	74.12 ± 0.74
10	66.69 ± 2.78
5	62.94 ± 2.44
0.5	67.93 ± 1.29
0.1	39.62 ± 1.45
0.05	22.38 ± 3.14

Therefore, allopurinol at a concentration of 100 µg/ml was used as the standard concentration throughout the analysis of XO inhibitory activity of distilled water extract of *Carica papaya* leaves.

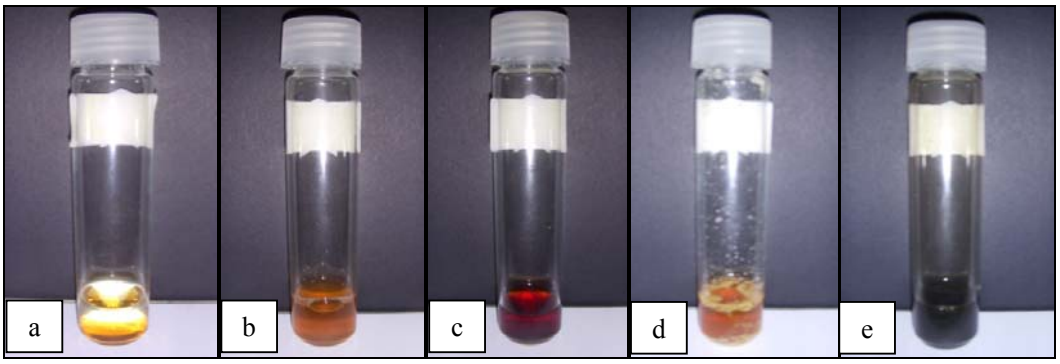

Fig. 1: Some of the secondary metabolites detected in the optimized crude extract of *Carica papaya* leaves using distilled water as the extraction solvent; (a) flavonoids, (b) alkaloids, (c) xanthine alkaloids, (d) saponins, and (e) anthranol glycosides, which may have contributed on the total XO inhibitory activity.

Quantitative Analysis of Carica papaya Leaves for their Phytoconstituents:

Quantitative determination of distilled water extract of *Carica papaya* leaves showed the presence of 27.51-33.15% flavonoids, 14.43-16.72% alkaloids and 1.12-1.97% saponins. Phytoconstituent at the highest percentage could be responsible for the XO inhibitory activity though their exact mode of inhibition is not yet understood. In the present study, the sample under evaluation possessed the highest percentage of flavonoids. Many classes and sub-classes of phenolic compounds have been reported to be a potent XOI such as polyphenols, flavonoids, coumarins, kaempferol and quercetin (Costantino *et al*., 1992; Chang and Chiang, 1995).

The Efficacy of TLC-RPFCC-HPTLC for the Purification of XOI:

The analytic fractionation of the distilled water extract of *Carica papaya* leaves using preparative TLC, eluted with M8 gave three separated constituents (MH1-MH3), M15 gave four separated constituents (EEA1–EEA4) and M18 gave three separated constituents (ME1-ME3). These three solvent systems were employed throughout the purification process separately. The flow rate for the RPFCC was fixed at 5 ml/min as it neither offers significant effect on the separations nor has any productivity advantages. HPTLC was employed after collection of liquid fractions isolated from flash column chromatography. Separated constituent appeared as a single spot on HPTLC were compared with the separated constituents from the preparative TLC. It was recovered by scraping off the adsorbent at the appropriate places on the developed plate and then by eluting the powder with a solvent and finally by centrifugation to remove adsorbent. There were ten constituents recovered from the HPTLC, similar to the number of constituents separated via preparative TLC. These ten constituents were subjected to bioassay to determine their inhibitory activity on XO and the activity was compared to the activity exhibited by the crude optimized extract and allopurinol as shown in Fig. 2. All fractions were tested at a concentration of 100 µg/ml, including the allopurinol. The results demonstrated that fractions EEA1 and EEA2 possessed high XO inhibitory activity exceeding the activity exhibited by the optimized crude extract of *Carica papaya* leaves with 95.70 ± 2.57% and 91.75 ± 3.12%, respectively. In fact, EEA1 have shown higher inhibition of XO as compared to allopurinol, a synthetic XOI. The other fractions in decreasing order of XO inhibition are EEA3 (84.75 ± 3.84%), ME1 (80.74 ± 1.33%), ME2 (70.31 ± 3.72%), ME3 (66.91 ± 4.77%), MH1 (58.90 ± 1.78%), MH2 (47.88 ± 4.91%), MH3 (43.44 ± 2.31%) and EEA4 (23.28 ± 0.25%). The polarity of the eluting

solvent has great contribution to the higher XO inhibitory activity. Polar solvents dissolve polar compounds more effectively than non-polar solvents and compounds with functional groups such as esters, alcohols, amines and acids elute successfully in polar solvents. Many polar compounds have been reported to be potent inhibitors of XO (Selloum *et al*., 2001; Cotelle *et al*., 1996; Unno *et al*., 2004; Costantino *et al*., 1992; Chang *et al*., 1993; Chang and Chiang, 1995) The constituents separated on the HPTLC appeared as a single spot, which suggests that they are present in pure form. Therefore, the purified constituent recovered from HPTLC is useful for further identification using preparative HPLC or FTIR.

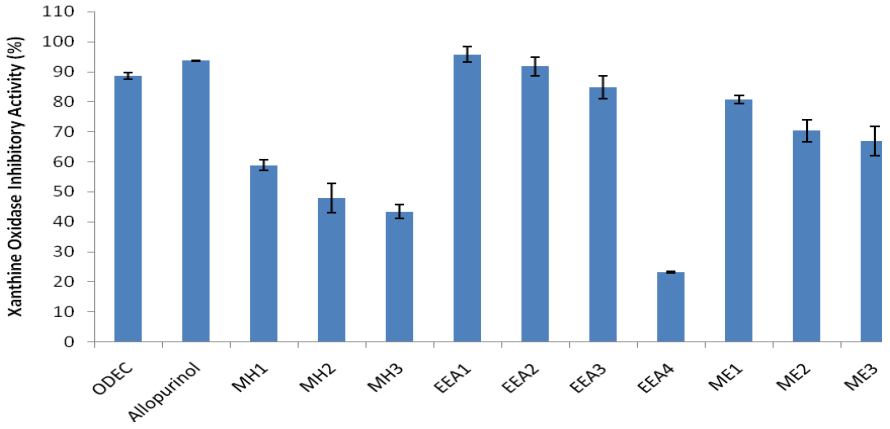

Fig. 2: Inhibition of XO by optimized distilled water extract of *Carica papaya* leaves (ODEC), allopurinol (synthetic XOI) and all ten fractions recovered from HPTLC at a final concentration of 100 µg/ml. MH as Methanol-Hexane, EEA as Ethanol-Ethyl Acetate and ME as Methanol-Ethanol. Each value is represented as mean ± S.D. from triplicate measurement.

Conclusion:

Carica papaya leaves extract has a bright future as a novel anti-gout agent by inhibiting XO, an enzyme that catalyzes the production of uric acid, subsequently responsible for the formation of gout, as well as in a variety of industrial applications including food ingredients, food supplements and other nutraceutical applications. The current attempt of purifying XOI compound via combination of methods of TLC-RPFCC-HPTLC serves as preliminary step for the development of new XOI from the plant origin. Further confirmation on the purity of the XOI compound obtained from *Carica papaya* leaves through identification of the active functional groups present using Fourier Transform Infrared Spectroscopy (FTIR) and by comparing with available standards. Identification of the active XOI compound using High Performance Liquid Chromatography (HPLC), determination of the structure of the active compounds as well as kinetic studies on the mode of enzyme activity inhibition warranted more attention. The production of anti-gout compound from Malaysian natural sources is very important for marketing in large scale. In addition it could be used as a promising substitute to the current irresponsive medicine.

ACKNOWLEDGEMENT

The research was supported by a research grant approved by the Research Management Center (RMC), International Islamic University Malaysia (IIUM). The authors are grateful to the RMC and Department of Biotechnology Engineering, IIUM for supporting and providing the laboratories facilities.

REFERENCES

Bhat, S.V., B.A. Nagasampagi and S. Meenakshi, 2008. Natural Products: Chemistry and Applications. Oxford: Alpha Science International Ltd.

Bickler, J.R., 2001. Natural product purification using flash chromatography. The Journal of American Association of Pharmaceutical Scientist, 9.

Bohm, B.A. and M.R. Koupai-Abyazani, 1994. Flavonoids and condensed tannins from leaves of Hawaiian *Vaccinium vaticulatum* and *Vaccinium calycinium*. Pacific Science, 48: 458-463.

Boyer, R., 1998. Concepts in Biochemistry. USA: Brooks/Cole Publishing Company.

Chang, W.S. and H.C. Chiang, 1995. Structure activity of coumarins in xanthine oxidase inhibition. Anticancer Research, 15: 1969-1974.

Chang, W.S., Y.J. Lee, F.J. Lu and H.C. Chiang, 1993. Inhibitory effects of flavonoids on xanthine oxidase. Anticancer Research, 13: 2165-2170.

Costantino, L., A. Albasini, G. Rastelli and S. Benvenuti, 1992. Activity of polyphenolic crude extracts as scavengers of superoxide radicals and inhibitors of xanthine oxidase. Planta Medica, 58: 342-344.

Cotelle, N., J.L. Bernier, J.P. Catteau, J. Pommery, J.C. Wallet and E.M. Gaydou, 1996. Anti-oxidant properties of hydroxyflavones. Free Radical Biology and Medicine, 20: 35-43.

Edeoga, H.O., D.E. Okwu and B.O. Mbaebie, 2005. Phytochemical constituents of some Nigerian medicinal plants. African Journal of Biotechnology, 4: 685-688.

Fransworth, N.R., 1966. Biological and phytochemical screening of plants. Journal of Pharmaceutical Sciences, 55: 225-276.

Harborne, J.B., 1973. Phytochemical methods: A guide to modern technique of plant analysis, 1st edition. London: Chapmann & Hall.

Harborne, J.B., 1984. Phytochemical methods: A guide to modern technique of plant analysis, 2nd edition. London: Chapmann & Hall, pp: 192.

Harborne, J.B., 1998. Phytochemical methods: A guide to modern technique of plant analysis, 3rd edition. London: Chapmann & Hall.

Janssens, H.J., E.H. van de Lisdonk, M. Janssen, H.J. van den Hoogen and A.L. Verbeek, 2006. Gout, not induced by diuretics? A case-control study from primary care. Annals of the Rheumatic Diseases, 65: 1080-1083.

Kokoglu, E., A. Belce, E. Ozyurt and Z. Tepeler, 1990. Xanthine oxidase levels in human brain tumors. Cancer Letters, 50: 179-181.

Lin, C-M., C-S. Chen, C-T. Chen, Y-C. Liang and J-K. Lin, 2002. Molecular modeling of flavonoids that inhibits xanthine oxidase. Biochemical and Biophysical Research Communications, 294: 167-172.

Marini-Bettolo, G.B., M. Nicoletti and M. Patamia, 1981. Plant screening by chemical and chromatographic procedure under field conditions. Journal of Chromatography, 218: 113-217.

McCord, J.M. and I. Fridovich, 1968. The reduction of cytochrome c by milk xanthine oxidase. The Journal of Biological Chemistry, 243: 5753-5760.

Naoghare, P.K., H.T. Kwon and J.M. Song, 2010. On-chip assay for determining the inhibitory effects and modes of action of drugs against xanthine oxidase. Journal of Pharm and Biomed Anal, 51: 1-6.

Naseem, S.A., F. Muhammad, H.N. Muzammil, B.M. Kouser and H. Aurangzeb, 2006. Activity of polyphenolic plant extracts as scavengers of free radicals and inhibitors of xanthine oxidase. The Journal of Basic and Applied Sciences, 2.

Obadoni, B.O. and P.O. Ochuko, 2001. Phytochemical studies and comparative efficacy of the crude extracts of some haemostatic plants in Edo and Delta States of Nigeria. Global Journal of Pure Applied Science, 8: 203-208.

Owen, P.L. and T. Johns, 1999. Xanthine oxidase inhibitory activity of Northeastern North American plant remedies used for gout. The Journal of Ethnopharmacology, 64: 149-160.

Perry, L.M. and J. Metzger, 1980. Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses. Cambridge: The MIT Press.

Ponce, A.M., S.E. Blanco, A.S. Molina, R. Garcia-Domenech and J. Galvez, 2000. Study of the action of flavonoids on xanthine oxidase by molecular topology. Journal of Chemical, Informational and Computational Science, 40: 1039-1045.

Selloum, L., S. Reichl, M. Muller, L. Sebihi and J. Arnhold, 2001. Effects of flavonols on the generation of superoxide anion radicals by xanthine oxidase and stimulated neutrophils. Archives of Biochemistry and Biophysics, 395: 49-56.

Shimoto, T., N. Ashizawa, K. Matzumoto, T. Nakazawa and O. Nagata, 2005. Simultaneous treatment with citrate prevents nephropathy induced by FYX-051, a xanthine oxidoreductase inhibitor, in rats. Toxicological Sciences, 87: 267-276.

Singh, V P., S.K. Sharma and V.S. Khare, 1980. Medicinal plants from Ujjain district Madhya Prades, Part II. Indian Drugs & Pharmaceutical Index, 5: 7-12.

Umamaheswari, M., K. Asok Kumar, A. Somasundaram, T. Sivashanmugam, V. Subhadradevi and T. K. Ravi, 2007. Xanthine oxidase inhibitory activity of some Indian medicinal plants. Journal of Ethnopharmacology, 109: 547-551.

Unno, T., A. Sugimoto and T. Kakuda, 2004. Xanthine oxidase inhibitors from the leaves of *Lagerstroemia speciosa* (L.). Pers. Journal of Ethnopharmacology, 93: 391-395.