International Scholars Journals

Advanced Journal of Environmental Science and Technology ISSN 2756-3251, Vol. 14 (1), pp. 001-004, January, 2023. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Exploration of xanthine oxidase producing microorganisms from hypoxanthine supplemented soil and their inhibitors from plants employing nitroblue tetrazolium

Jyoti Mahajan, Rakesh Kumar, Rajbir Kaur and Saroj Arora*

Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar-143005, Punjab, India

Accepted 20 March, 2022

Xanthine oxidase is a member of molybdoenzyme family and has catalytic role in purine degradation, metabolizing hypoxanthine and xanthine to uric acid with the generation of superoxides. In the present study, microtitre plate based colorimetric assay was used to screen the xanthine oxidase producing microorganisms from the soil treated with hypoxanthine with different concentrations (2mM, 4mM, 6mM) for six weeks daily and at an interval of seven days. The basic principle of assay is that the superoxides produced by microbial cultures, grown in xanthine rich medium interacts with Nitroblue tetrazolium (NBT) solution and produces dark blue color, showing the presence of xanthine oxidase. Furthermore, the plant extracts were assessed for their xanthine oxidase inhibitory activity. The inhibitory effect of polyphenolic rich fractions of *Juniperus recurva* and *Chukrasia tabularis* was determined to find out the natural inhibitors of xanthine oxidase. The inhibitory effect of allopurinol on xanthine oxidase was also observed.

Keywords: Hypoxanthine, Allopurinol, Xanthine Oxidase, Nitroblue Tetrazolium.

INTRODUCTION

Xanthine oxidase is a cytoplasmic enzyme, converting hypoxanthine to xanthine and generates superoxides and uric acid. It is commercially important enzyme, extracted from fresh milk. It is also used as an auxillary enzyme in the staining of several enzymes or tissues. It can also control the gout attacks and these attacks can be treated with the use of antihyperuricaemic therapy either with uricosuric drugs or xanthine oxidase inhibitors. Xanthine oxidase is capable of oxidizing many varieties of purines and also the enzyme tissue generator of superoxide ions which react with hydrogen peroxide to generate hydroxyl radicals. The main site of xanthine oxidase is found in the liver and intestinal lining in humans. It has low substrate specificity and catalyzes the reaction between xanthine, hypoxanthine or acetaldehyde with molecular oxygen. Lin et al., (2002) studied the molecular modeling of flavonoids that inhibit xanthine oxidase and reported the apigenin as the most potent inhibitor. Takahama et al., (2010) reported the inhibition of xanthine oxidase activity by an oxathiolanone derivatives of quercetin. Many investigators have studied the mechanisms of inhibition and reported that the C_5 and C_7 hydroxyl groups have an important role in the inhibition (Bindoli et al., 1985; Nagao et al., 1999). Furthermore, oxathiolanone derivative of rutin-oxathiolanone have structural similarity of xanthine, allopurinol and oxypurinol and leads to exhibit xanthine oxidase activity more effectively than their mother compounds. Allopurinol is generally used in the management of gout and conditions associated with hyperuricemia but its adverse effect leads to GIT distress, hypersensitivity reactions and hepatitis. The toxicity of allopurinol also leads to symptoms like fever, rashes, vasculitis, eosinophilia and worsening of renal function. Quisumbing (1978) interrogated the herbal finders who trade fresh plants and preparations for various conditions ranging from fever to abortifacients. There are thousands of herbal plants that had attributed medicinal benefits.

*Corresponding author E-mail: sarojarora.gndu@gmail.com; Phone: +91-183-2451048, +91-9417285485; Fax: +91-183-2258819, 2258820

However, a considerable number of plants still needs to be scientifically validated and much work is needed to investigate the bioactivity and phytochemicals of these plants. Several plant extracts from China, North America, Brazil and Australia have shown inhibitory effects on xanthine oxidase (Kong et al., 2000; Owen and Johns., 1999; Filha et al., 2006; Sweeney et al., 2001). Therefore, there is urgent need to search the inhibitors of xanthine oxidase from the plant sources.

METHODS

Chemicals

Hypoxanthine, Allopurinol, Nitroblue tetrazolium, Tris-HCl buffer, Sodium hydroxide, Sodium chloride, Sodium phosphate, Potassium phosphate, Ammonium chloride.

Microbial culture

Seven plots (1×1 feet) in the Botanical garden of Guru Nanak Dev University, Amritsar, India was selected for the augmentation of hypoxanthine utilizing microorganisms in the soil. The soil was enriched by spraying with different concentrations of hypoxanthine solutions (2mM, 4mM, 6mM). The treatments were given daily and weekly for six weeks on the selected soil area and samples were collected after a week. The spraying of hypoxanthine solution and collection of soil samples were continued for six weeks. In total, forty two samples were collected.

Preparation of soil suspension

After the collection of soil samples from designated area, suspension was prepared for each sample (1g) by adding 10 ml of tap water followed by vortexing of the solution.

Inoculation

After the vortex process, 1 ml of supernatant was used as an inoculum in 50 ml of Minimal salt medium (sodium chloride, sodium phosphate, potassium phosphate and ammonium chloride) containing hypoxanthine and incubated at 37°C in an orbital shaker (200 rpm) for six days.

Maintenance and purification of the isolates

After the completion of incubation period, the samples were streaked on the MSM agar plates containing hypoxanthine and placed in BOD incubator at 37°C for three days. The positive isolates were selected and purified on nutrient agar plates and incubated for 24 hours.

Nitroblue Tetrazolium assay

The Nitroblue tetrazolium assay was carried out to determine the xanthine oxidase activity in microbial cultures by following the methodology of Ozer et al., (1998). For this, microbial culture of different concentrations were grown overnight in Luria Broth medium. These cultures were centrifuged (2000 rpm, 8 minutes) and the pellet was suspended in 1 ml of Tris-HCl buffer (50 mM, pH= 7.6). The microbial cultures were lysed with 250 µl of NaOH followed by the addition of NBT (1 ml, 0.5mM) solution. The reaction mixture was incubated for five minutes at ambient temperature and then centrifuged at 2000 rpm for eight minutes and absorbance was recorded at 575nm.

Control Experiments

NBT assay was set up by control experiments. First control experiment consist of 1 ml of NBT and 1 ml of NaOH solutions, known as negative control as there was no color development. Second control experiment was carried by taking 500 µl of microbial culture, 1.5 ml of allopurinol and then the reaction mixture was incubated for five minutes at ambient temperature and after that 500 µl of NBT, 1.5 ml of NaOH was added and again incubated for five minutes. It was centrifuged and absorbance of supernatant was recorded at 575 nm.

RESULTS AND DISCUSSION

The results of different experiments are shown in Table 1, 2 and 3. The hypoxanthine was sprayed daily and at an interval of seven days continuously for six weeks in both the cases. The soil samples were collected after seven days for six weeks along with the samples without the spray of hypoxanthine. In total, forty two soil samples were screened for xanthine oxidase activity. In the present study, some of the microbial cultures containing hypoxanthine reacts with NBT solution and give dark blue color in the dose dependent manner, showing the presence of xanthine oxidase. It was further observed that the xanthine oxidase activity in different samples varied from good to moderate to negligible amount. There was no color development in the negative control. It was also noticed that the light blue color was developed in the microbial cultures which were treated with allopurinol (known inhibitor of xanthine oxidase). The increased

Table 1. Absorbance of microbial cultures on daily and weekly basis from first to sixth week of hypoxanthine treatment

Hypoxanthine Concentration (mM)	ABSORBANCE OF MICROBIAL CULTURES											
	Ist Week		IInd Week		IIIrd Week		IVth Week		Vth Week		VIth Week	
	D	W	D	W	D	W	D	W	D	W	D	W
0	0.012		-0.016		0.03263		0.0538		0.0193		0.405	
2	0.073	0.721	-0.007	0.169	0.8566	0.5063	0.8	0.574	0.426	0.594	0.611	0.445
4	0.223	0.742	0.238	0.083	0.467	0.7896	0.409	0.509	0.389	0.755	0.549	0.855
6	0.442	0.809	0.012	0.129	1.03	1.457	-0.032	0.398	0.45	0.674	0.488	0.896

D* = Daily
W*= Weekly

Table 2. Inhibition percentage of allopurinol from first to six week of hypoxanthine treatment

Hypoxanthine Concentration(mM)	INHIBITION PERCENTAGE											
	Ist Week		IInd Week		IIIrd Week		IVth Week		Vth Week		VIth Week	
	D	W	D	W	D	W	D	W	D	W	D	W
2	14.65	83.48	60.25	20.11	83.13	77.48	71.5	70.05	98.04	81.20	7	44.15
4	49.6	47.49	60.27	38.22	78.21	86.57	61.44	42.47	62.39	37.43	28.80	31.95
6	65.86	32.06	39.31	62.65	81.43	93.63	53.21	33.66	93.10	92.45	49.88	72.39

D* = Daily
W*= Weekly

Table 3. Comparison of inhibition of different plant extracts on the activity of xanthine oxidase treated with different concentrations of hypoxanthine

Week	Concentration (mM)	Spray	Culture absorbance (nm)	Plant extract absorbance(nm) A*	B *	C*	D*
First	4	Daily	0.223	0.656	0.8430	0.768	0.571
Second	4	Daily	0.238	0.308	0.4430	0.224	0.138
Third	6	Daily	1.03	0.326	0.4806	0.239	0.147
Fourth	2	Daily	0.8	0.287	0.3305	0.888	0.275
Fifth	4	Daily	0.389	0.374	0.3809	0.143	0.184

A*= *Chukrasia tabularis* methanol plant extract
B*= *Juniperus recurva* methanol plant extract
C*= *Juniperus recurva* chloroform plant extract
D*=*Juniperus recurva* methanol fraction plant extract

absorbance indicates the increased amount of formazan formed due to the excess of superoxides produced during the reduction of oxygen by microbial xanthine oxidase. However, when allopurinol was added to the reaction mixture, color intensity decreased that results to decrease in the concentration of superoxides radical as allopurinol inhibited the xanthine oxidase activity and hence free radical formation. Appearance of slight blue color can be attributed to the production of superoxides by other internal factors like mitochondrial electron chain and many autooxidation reactions. In case of the microorganisms which were lacking the xanthine oxidase activity showed no difference in absorbance of the cultures without allopurinol or the cultures containing allopurinol indicating that these microorganisms do not have the xanthine oxidase activity. Although, the xanthine oxidase/dehydrogenase system has been extensively studied with the bovine milk oxidase and chicken and rat dehydrogenase and has become the standards of comparisons for xanthine oxidizing enzymes isolated from other sources but microbial xanthine oxidase can be exploited to address a number of questions presently not possible with eukaryotic enzymes (Woolfolk., 1978; Sato et al., 1995). These preliminary studies although precludes the detection of alloenzymes or isozymes multiplicity but this assay method can pave the way to increase the efficiency of screening libraries of microorganisms for xanthine oxidase activity and also can be exploited for the screening of xanthine oxidase inhibitors. In the present study, the plant extracts were

used to find out the inhibitory effect on the activity of xanthine oxidase that also affects or inhibits the production of superoxide anions. *Chukrasia tabularis* and *Juniperus recurva* were used to find out their effect on xanthine oxidase and it has been found that the methanol fraction of *J. recurva* have more inhibition against xanthine oxidase activity in the samples treated with different concentrations of hypoxanthine on second, third, fourth and fifth weeks as compared to other plant extracts. The methanol extract of *C. tabularis* showed inhibition of xanthine oxidase activity on microbes isolated from soil samples collected after third week of hypoxanthine treatment (6mM). A similar trend was observed in microbes from fourth week and fifth week of hypoxanthine treated soil samples. However, the methanol extract did not show the inhibition of xanthine oxidase activity in soil samples collected after first and second week of treatment. The effect of methanol extract of the *Juniperus* on the xanthine oxidase activity in microbes isolated from first five weeks treated soil with hypoxanthine was seen and it has been found that the extract showed the xanthine oxidase inhibitory activity. A similar trend was observed in microbes isolated from soil samples treated with hypoxanthine for four weeks. The *Juniperus* chloroform extract exerted inhibitory action on the xanthine oxidase activity on third week samples with reduction in absorbance from 1.03 nm to 0.239 nm.

ACKNOWLEDGEMENTS

The authors are thankful to University Grants Commission (UGC), New Delhi for providing financial assistance. We are also thankful to Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar for providing necessary laboratory facilities.

REFERENCES

Bindoli A, Valente M, Cavallini L (1985). Inhibitory action of quercetin on xanthine oxidase and xanthine dehydrogenase activity. Pharmacol. Res. Communications, 17: 831–839.

Filha ZS, Vitolo IF, Fietto LG, Lombardi JA, Saude GDA (2006). Xanthine oxidase inhibitory activity of *Lychnophora* species from Brazil ("Arnica"). J. Ethnopharmacol., 107: 79–82.

Kong LD, Cai Y, Huang W, Cheng C, Tan R (2000). Inhibition of xanthine oxidase by some Chinese medicinal plants used to treat gout. J. Ethnopharmacol., 73: 199-207.

Lin CM, Chen CS, Chen CT, Liang YC, Lin JK (2002). Molecular modeling of flavonoids that inhibits xanthine oxidase. Biochem. and Biophy. Res. Communications, 294: 167–172.

Nagao A, Seki M, Kobayashi H (1999). Inhibition of xanthine oxidase by flavonoids. Biosci. Biotechnol. and Biochem. 63: 1787–1790.

Owen P, Johns T (1999). Xanthine oxidase inhibitory activity of northeaster North American plant remedies used for gout. J. Ethnopharmacol., 64: 149-160.

Ozer N, Oglu MM, Ogus IH (1998). A simple and sensitive method for the activity staining of xanthine oxidase. J. Biochem. and Biophy. Methods, 36: 95-100.

Quisumbing E (1978). Medicinal plants of the Philippines. Katha Publishing. P. 1262.

Sato A, Nishino T, Noda K, Amaya Y, Nishino T (1995). The structure of chicken liver xanthine dehydrogenase cDNA cloning and the domain structure. J. Biol. Chemist, 270: 2818-2826.

Sweeney AP, Wyllie SG, Halliker RA, Markham JL (2001). Xanthine oxidase inhibitory activity of selected Australian native plants. J. Ethnopharmacol., 75: 273-277.

Takahama U, Koga Y, Hirota S, Yamauchi R (2010). Inhibition of xanthine oxidase activity by an oxathiolanone derivative of quercetin. Food Chemistry, 126: 1808-1811.

Woolfolk CA, Downard JS (1978). Bacterial xanthine oxidase from Arthrobacter S-2. J. Bacteriol., 135: 422–428.