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Research Article

Pyrogallol is a key component for xanthine oxidase inhibition by the leaves of *Ammannia baecifera*

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Abstract

Ammannia baecifera Linn is an important plant in traditional medication system of Bangladesh. In order to give the scientific clarification for using this plant in the treatment of gout, this study was designed to identify the xanthine oxidase (XO) inhibitors present in the extract of *Ammannia baecifera* leaves along with evaluating its antioxidant effect. First aqueous extract was prepared from leaves and then water insoluble part (WIP) was separated from water soluble part (WSP) as precipitate with ethanol. In DPPH and ABTS assays, WSP with rich amount of phenolic compound exhibited high radical scavenging activity than WIP. WSP also showed potent XO inhibition with IC_{50}: 18.03 ± 1.65 µg/mL value. Using different chromatographic techniques, the key XO inhibitor was isolated from WSP and identified as pyrogallol based on its different spectroscopic data. In XO inhibition assay, pyrogallol (IC_{50}: 11.67 ± 0.45 µg/mL) showed potent inhibitory effect as compared to a known XO inhibitor (allopurinol; IC_{50} = 27.35 ± 1.15 µg/mL) and it also displayed strong DPPH and ABTS radical scavenging effect. In addition, an inhibition kinetics study indicated that WSP and pyrogallol are mixed competitive inhibitors of XO. The overall results suggest that the presence of pyrogallol as XO inhibitor and free radical scavenger in *Ammannia baecifera* leaf justifies the traditional uses of this plant.

Keywords: *Ammannia baecifera*, Leaves, Xanthine oxidase, Pyrogallol, Inhibitor

INTRODUCTION

In human body, xanthine oxidase (XO) plays important role in catabolic pathways of purine metabolism and it present in the gastrointestinal tract and liver with significant concentrations. XO is involved in the formation of reactive oxygen species like superoxide and hydrogen peroxide when it catalyzes the oxidation reaction of hypoxanthine and xanthine [1]. These reactive oxygen species act as the leading contributor for causing the oxidative damage of living tissues which ultimately accelerates the formation of several physiological disorders such as diabetes mellitus, heart diseases, cancer, aging, Parkinson's disease, Alzheimer's disease, renal failure and so on [2, 3]. XO is also responsible for development of gout where excessive uric acid deposition in the joints cause painful inflammation [4]. Therefore, the selective inhibition of XO might be a broad-spectrum therapeutic tool to treat gout, oxidative damages and inflammatory-associated diseases. As a clinically recognized XO inhibitor, allopurinol is used to treat gout. However, different adverse effects of this drug such as nephropathy, numbness, tingling, burning pain and allergic reactions, limits its uses as a drug [4, 5]. Thus, it is a demand of time to develop novel XO inhibitors that have a higher therapeutic activity and fewer side effects. In this context, plant materials have the merit for taking them as the valuable source of secondary metabolites with antioxidant and XO activities [6]. In this study we have conducted screening on the leaves of *Ammannia baecifera* for XO inhibitors.

Ammannia baecifera Linn. belonging to the family Lythraceae, is a herbaceous weed of wet places and grows commonly in rice field and marshy places throughout Bangladesh [7]. Traditionally this plant is used in the treatment of rheumatic pains, glandular swellings, leucorrhea, abscesses, ulcers, polyuria and fevers. *Ammannia baecifera* has a wide range of therapeutic and pharmacological properties, including antibacterial, antifungal, anti-depressant, antiurolithiasis, antitumor, anti-inflammatory, antioxidant, analgesic and antisteroidogenic activities [8, 9, 10, 11, 12]. From the information available so far on the beneficial effects of *Ammannia baecifera* extract, it is clear that most of the secondary metabolites identified in this plant are phenolic in nature and these metabolites are responsible for its various types of biological activities [13, 14]. In addition, leaves of *Ammannia baccifera* have also been used for the treatment of pain in joints but so far there are no scientific reports available on its inhibitory potentials against XO. So here leaves of *Ammannia baccifera* were evaluated for free radical scavenging activity and the inhibitory potential against xanthine oxidase (XO) to clarify this folkoric activity.

MATERIALS AND METHODS

Chemicals

2,2´-diphenyl-1-picrylhydrazyl (DPPH•), 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Sephadex® G-25 were purchased from sigma-aldrich

(USA) whereas gallic acid, Folin-ciocalteu reagent (FCR), potassium persulfate and aluminum chloride ($AlCl_3$) were obtained from Carl Roth (Germany). Ethanol, dimethyl sulfoxide (DMSO), xanthine, xanthine oxidase, pyrogallol and allopurinol were purchased from Fujiflim Wako Chemicals Corporation (Japan).

General experimental procedures

The spectrophotometric reading was taken on HALO RB-10 (Wavelength range: 190-1000 nm), Dynamica, Australia whereas Varioskan multimode reader with Flash spectral scanning, Thermo Scientific, USA (Monochromators with double excitation and emission mode and wavelength range: 200 -1000 nm) were used here as ELISA microplate reader. Bruker AVANCE 500 spectrometer was used to take NMR spectra and sample was dissolved in D_2O that produce residual solvent signal at δ 4.80 for ^1H. Mass spectra were taken using a Benchtop ESI-TOF mass spectrometer of Bruker where fragmentation was conducted on a compact qTOF mass spectrometer with multiple reaction monitoring operational mode.

Plant material collection and authentication

Ammannia baccifera Linn. leaves had been collected from the Meherchandi area adjacent to Rajshahi University campus of Rajshahi district in January, 2020. A taxonomist of Botany Department (Professor Dr. A. H. M. Mahbubur Rahman) in University of Rajshahi had authenticated this plant and then it was deposited in the herbarium of this department under the specimen record number of 943. After washing with tap water, the collected plant materials were left to dry at room temperature. Finally dried leaves were ground into powder by a grinder machine and preserved in glass containers for further use.

Preparation of plant extract

20 g of leaf powder was soaked in 500 mL distilled water for 48 hrs in dark condition and at 37°C temperature. After 48 hrs, the extract was prepared through filtration using a filter paper and it was concentrated with a rotary evaporator. Then the water insoluble part (WIP) in concentrated extract was removed as precipitate with ethanol to have 2.3 g. Then 4.4 g water soluble part (WSP) was obtained through freeze drying. Both water soluble (WSP) and insoluble (WIP) parts were preserved in glass vials at -80°C for various analyses.

Estimation of total polyphenolic content

The previously described FCR based method was used to estimate the total phenolic content (TPC) of WSP and WIP where gallic acid was taken as a standard phenolic compound [15]. Concisely, subsequent addition of 6 mL distilled water, 0.5 mL of FCR reagent (10 times diluted) solution and 1 mL of sodium carbonate (5%) solution to the test tubes that previously contained 1 mL of samples/standard solution of different concentrations. After adjustment of the volume of each test tube to 10 mL and incubation for 60 min at room temperature, the absorbance was measured in spectrophotometer at 760 nm. Then the standard curve was built for gallic acid (GAE) and the total phenolic content of the extracts (WSP and WIP) was calculated and expressed as gallic acid equivalents per gram of sample dry weight.

Determination of DPPH radical scavenging activity

The DPPH radical scavenging properties of WSP, WIP, pyrogallol and ascorbic acid (used here as standard) were evaluated by the assay protocol described previously where stable radical DPPH was utilized to measure hydrogen donating or radical-scavenging ability of test samples [16]. Briefly, 3.0 mL of 0.1 mM DPPH solution in methanol was added to 1.0 mL of sample solution in water at different concentrations. After shaking, these reaction mixtures were kept in the dark place for 30 minutes at room temperature and then the absorbance of each resulting mixture was measured at 517 nm. Finally the following formula was used to calculate the percentage scavenging activity:

$$\%\text{inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where the absorbance of reaction mixture without samples was taken as $A_{control}$ and A_{sample} was the absorbance of the reaction mixture with samples. After three-time replication, the mean values were used to draw graph from which the EC_{50} values (μg/mL) were determined. Here EC_{50} value is the effective amount of the sample that is wanted to scavenge DPPH radical by 50%.

Determination of ABTS•+ scavenging activity

The previously described method was applied to evaluate the ABTS• radical scavenging properties of WSP, WIP, pyrogallol and standard (ascorbic acid) [17]. First 7 mM ABTS• stock solution was mixed with 2.45 mM potassium persulfate to achieve ABTS•+ radical and the mixture was incubated in the dark chamber for 12–16 hours at room temperature. An absorbance at 734 nm of 0.70±0.02 was adjusted by diluting the ABTS•+ solution with water. Then 3 mL of ABTS•+ solution was added to the test tubes containing test sample of different concentrations and finally the absorbance was taken at 734 nm after 6 minutes. Then the percentage scavenging activity and EC_{50} for test samples were determined using same way as described in DPPH• assay.

Xanthine oxidase inhibition assay

The formerly reported process was used to study the XO inhibitory activity of different samples where uric acid formation was monitored in a XO system [18, 19, 20]. Here 4 mM xanthine (substrate) and XO (enzyme) solution (0.16 U/mL) in 0.1M phosphate buffer (pH 7.6) were prepared immediately before use. Assays were accompanied using in a 96-well microplate. Here four type of reaction mixtures were prepared by the following way and leveled as sample, blank1, blank2 and blank3.

Sample: 20 μL sample + 125 μL xanthine solution + 20 μL enzyme solution + 50 μL 0.1M phosphate buffer

Blank1: 125 μL xanthine solution + 20 μL enzyme solution + 70 μL 0.1M phosphate buffer

Blank2: 125 μL xanthine solution + 90 μL 0.1M phosphate buffer

Blank3: 20 μL sample + 125 μL xanthine solution + 70 μL 0.1M phosphate buffer

The reaction mixture was incubated at 37°C for 25 minutes and absorbance was measured at 295 nm using ELISA microplate reader. The percentage inhibition was calculated according to the following equation:

$$\%\text{ inhibition} = 100 - [(\text{Sample} - \text{Blank3}) / (\text{Blank1} - \text{Blank2})] \times 100$$

Fractionation and isolation

Based on the XO inhibitory potential, WSP (0.66 g) was applied on gel filtration chromatography using G-25 gel as stationary phase and water as mobile phase. Fifteen fractions (volume of each fraction = 40 mL) were collected. Then three fractions (fr-6, 7 & 8) were combined together based on their XO inhibitory profile and this combined fraction was concentrated through freeze drying to provide 15 mg of dried material containing the target compounds. It was fractionated by preparative high performance liquid chromatography

(HPLC) using a COSMOSIL 2.5πNAP Packed Column (Nacalai Tesque Inc., 10 × 250 mm, 2 mL/min, UV detection at 254 nm) with a mixture of MeCN and 0.1% HCO_2H solution. Eight fractions were collected and fraction-5 (RT: 9.25 mins) was highly active against XO. Based on ^{1}H-NMR of fraction-5, it was again fractionated by preparative HPLC using the same condition and procedure used for the previous combined fraction and four fractions were collected. Among the four fractions, 1.5 mg white powder was found as fraction-5c which showed high inhibitory effect against XO. Finally the structure of the compound present in fraction-5c was analyzed by HR-ESITOF-MS, ^{1}H- and ^{13}C-NMR spectra. As a flow diagram, the overall purification process is shown in figure 1.

Kinetic studies of XO inhibition

The type of inhibition carried out by WSP, pyrogallol and allpurinol against XO was determined by the Lineweaver-Burk plot [21]. First 125 µL of xanthine solution (25, 50, and 100 µM) and 20 µL of sample solution (0, 3.12 and 6.25 µg/mL) were mixed in 96-well microplates and incubated at 37°C for 25 min. Then freshly prepared enzyme solution (0.10 U/mL in 0.1M phosphate buffer; pH 7.6) was added to the mixture to start the reaction and ELISA reader was used to record the absorbance at 295 nm for every 30 seconds up to 4 min. Finally, the Lineweaver-Burk Plots were drawn from the data obtained from the enzyme assays using Excel (Microsoft Office 2010).

Statistical Analysis

Data were presented as mean ± SD (Standard Deviation). One way analysis of variance (ANOVA) was used to conduct statistical analysis using SPSS statistical software of 16 version. Independent-sample T-test was applied to separate the means and statistically significant was considered at the level of $P<0.05$.

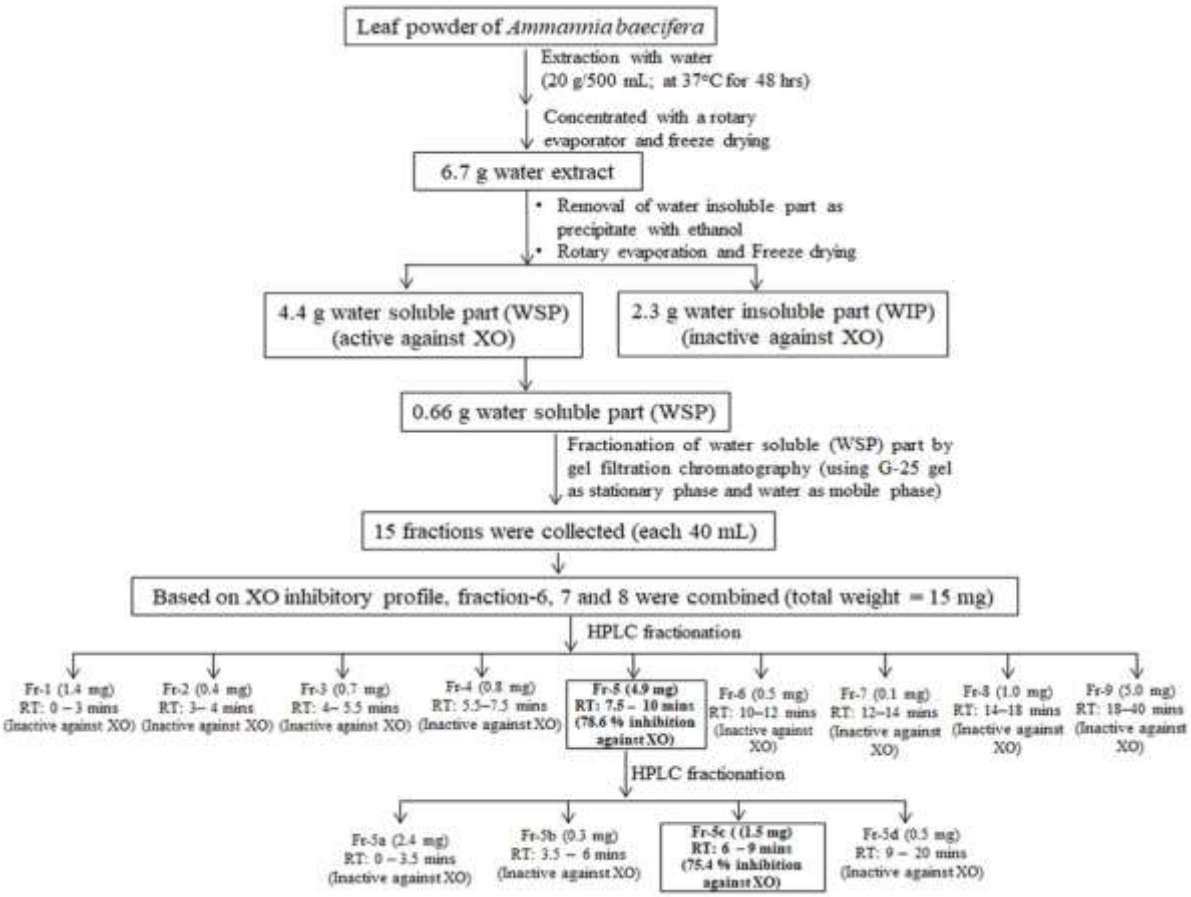

Figure 1: Scheme applied for purification of key component that act as XO inhibitor of *Ammannia baecifera* leaf

RESULTS

Total phenolic content of *Ammannia baecifera* leaf

The total amount of phenolic content present in WSP and WIP is shown in figure 2. In one gram of water soluble part (WSP), 78 ± 2.16 mg gallic acid equivalent of phenolic content was found and water insoluble part (WIP) had 20.99 ± 1.185 mg gallic acid equivalent of phenolic content.

Free radical scavenging activity of *Ammannia baecifera* leaf

In case of DPPH radical scavenging assay, the EC_{50} values of WSP, pyrogallol and WIP were 29.5 ± 1.78, 8.30 ± 0.78 and 251.7 ± 5.02 µg/mL, respectively (Table 1) whereas the EC_{50} values were found to be 16.9 ± 1.03, 6.25 ± 0.57 and 110.9 ± 3.15 µg/mL for WSP, pyrogallol and WIP, respectively in ABTS radical scavenging assay (Table 1).

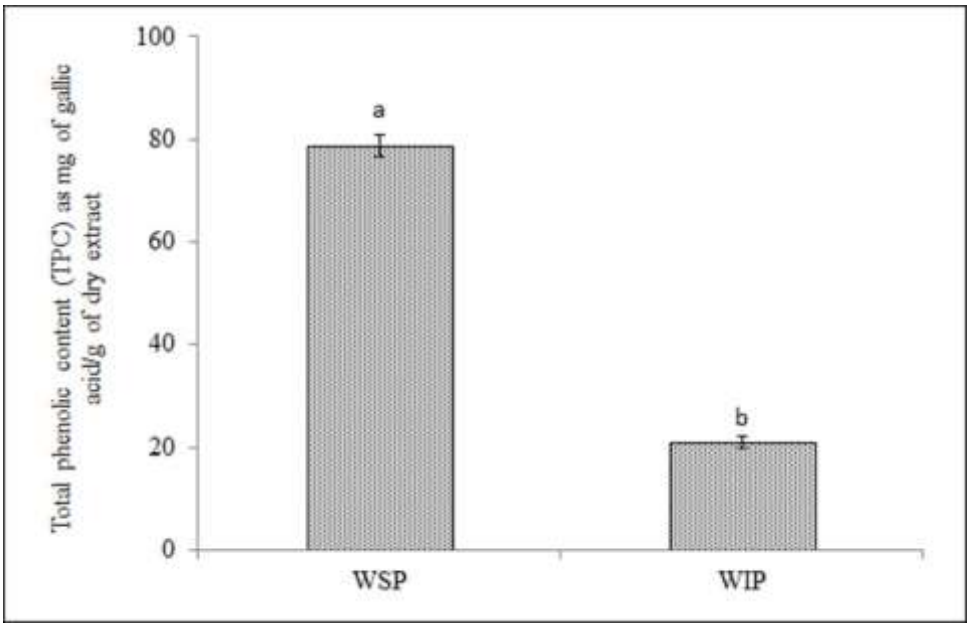

Figure 2: Total phenolic contents of WSP and WIP of *Ammannia baecifera* leaf. Value with different letter (a, b) are significantly different at *P*<0.05

Table 1: Free radical scavenging activity of *Ammannia baecifera* leaf

Samples	DPPH radical scavenging activity (IC_{50} in µg/mL)	ABTS radical scavenging activity (IC_{50} in µg/mL)
WSP	29.5 ± 1.78[a]	16.9 ± 1.03[a]
WIP	251.7 ± 5.02[b]	110.9 ± 3.15[b]
Pyrogallol	8.30 ± 0.78[c]	6.25 ± 0.57[c]
Ascorbic acid	5.35 ± 0.84[c]	6.19 ± 0.25[c]

Data are expressed as mean ± SD (Standard deviation). WSP: Water soluble part; WIP: Water insoluble part. Values in the same column with different letters (a, b, c) are significantly different at P < 0.05.

XO inhibitory activity of extracts and fraction of *Ammannia baecifera* leaf

The overall extraction, fractionation and purification procedure performed in this study for *Ammannia baecifera* leaf powder is presented in figure 1. Here water insoluble part (WIP) was separated from crude water extract of *Ammannia baecifera* leaf as precipitate with ethanol and the remaining part was used as water soluble part (WSP). In inhibition assay, WSP displayed high inhibitory activity against XO and the IC_{50} value was found to be 18.03 ± 1.65 µg/mL as shown in figure 3(a). But here WIP was inactive against XO. The WSP were then applied on gel filtration chromatography and 15 fractions were collected. Among these fractions, fraction-6, 7 and 8 showed high activity against XO and exhibited 19.4%, 88.3% and 63.9% inhibition, respectively. These three fractions were combined and again fractionated by HPLC to have nine fractions. After fraction with HPLC, only fraction-5 showed 75.4% inhibition against XO. Fraction-5 was further fractionated by HPLC and four fractions were collected. Then fraction-5(c) showed 78.6% inhibition against XO and contained the target inhibitor.

Identification of key XO inhibitor in *Ammannia baecifera* leaf

The fractionation of WSP by gel filtration chromatography and HPLC yielded 1.5 mg white powder as fraction-5c which was the key inhibitor of XO present in the leaf of *Ammannia baecifera*. The structure of this inhibitor was analyzed using the data of HR-ESI-MS and NMR. Its ESI-MS showed a molecular ion (M^+) peak at m/z 126.0317. Finally, it was identified as pyrogallol (figure 4) by a comparison of its ^{1}H- and ^{13}C-NMR data (Table 1) with the literature [22].

Table 2: ^{1}H- and ^{13}C-NMR spectral data of fraction-5c

Carbon No.	Fraction-5c (Dissolved in D_2O)	
	δ_C	δ_H
1	133.1	-
2	146.1	-
3	109.9	6.794 (t, 1H, *J* = 5.3 Hz)
4	120.4	6.586 (d, 1H, *J* = 5.3 Hz)
5	109.9	6.794 (t, 1H, *J* = 5.3 Hz)
6	146.1	-

Proton resonance integral, multiplicity, and coupling constant (*J* = Hz) are in parentheses.

XO inhibitory activity of WSP, Pyrogallol and Allopurinol

In this study, pyrogallol was identified as a key XO inhibitor present in WSP of *Ammannia baecifera* leaf. So, we had evaluated the XO inhibition efficacy (IC_{50}) for commercially purchased pyrogallol and compared with allopurinol (figure 3). The IC_{50} for pyrogallol (11.67 ± 0.458 µg/mL) was found to be lower than allopurinol (27.35 ± 1.15 µg/mL) thereby indicating its high inhibition potentials against XO.

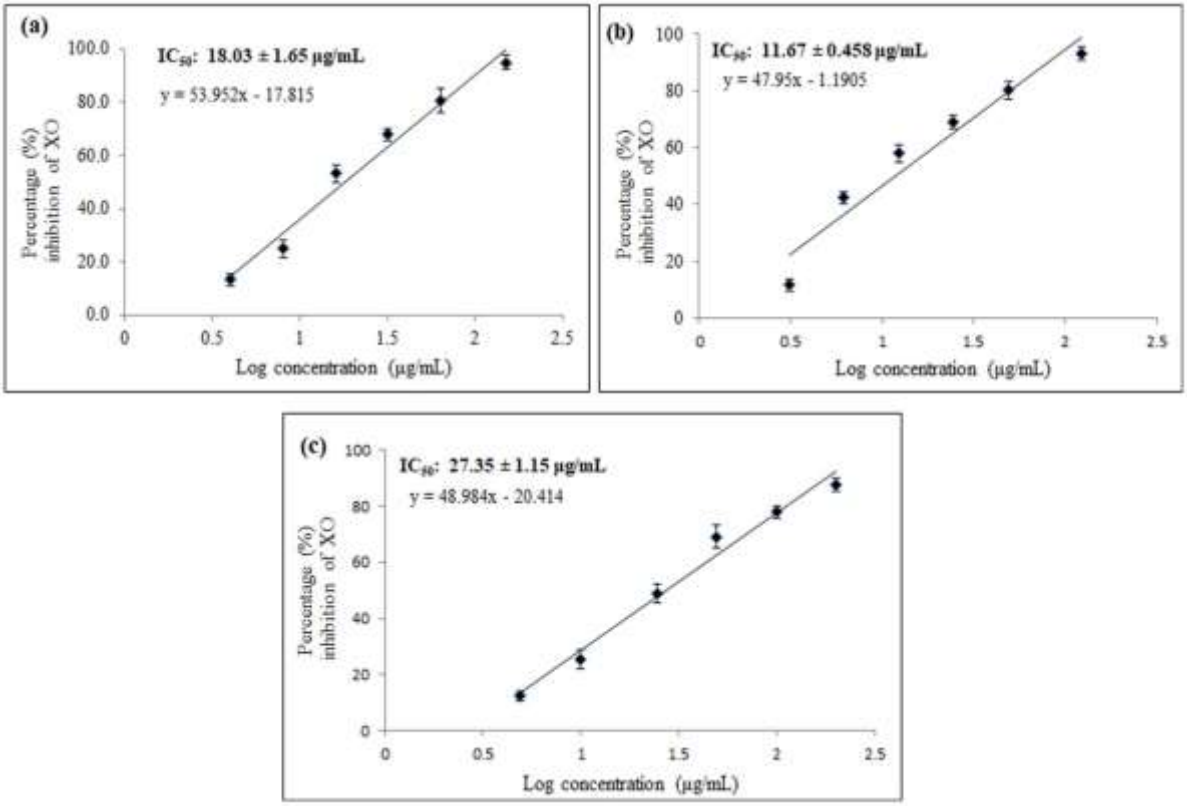

Figure 3: XO inhibitory activity of WSP (a), pyrogallol (b) and Allopurinol (c). Each value is presented as the mean ± standard deviation from triplicate measurements.

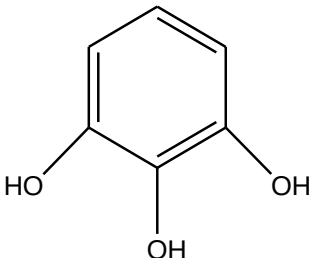

Figure 4: Chemical Structure of pyrogallol

DISCUSSION

Several studies have shown that indigenous antioxidants play vital role in preventing the lethal consequences of oxidative stress. That's why scientists are focusing on natural antioxidants due to their protective effect on biochemical functions against oxidative stress. In terms of this reality, phenolic compounds from plant sources are considered as an important reservoir of natural antioxidants [23]. In this study, WSP contained significantly high amount of phenolic content in respect to WIP. Similar pattern was observed in findings of previous studies where *Cinnamomum osmophloeum* twigs and olive leaves were evaluated [24].

DPPH and ABTS radicals scavenging assays are the two most widely used methods for determining comparative antioxidant effectiveness of plant extracts [25]. Here we have found that WSP of *Ammannia baecifera* leaf and pyrogallol isolated from WSP exhibited stronger scavenging capability in respect to WIP in DPPH and ABTS assays. Although both samples had a weaker scavenging ability at the low concentrations but the scavenging effect was increased with increment in concentrations. Positive results along with low EC_{50} in DPPH and ABTS tests confirm the presence free radical scavengers in samples [26]. Since WSP had high amount of polyphenolic content than WIP, so findings of this study was consistent with the previous report where aqueous leaf extract of *Stevia rebaudiana* containing high amount of polyphenolic compounds displayed more powerful radical scavenging properties [27]. Moreover, pyrogallol as one of the identified phenolic constituents of WSP, was found to be highly active free radical scavenger as compared to ascorbic acid (used as standard) and this was consistent with the results obtained in previous studies where pyrogallol exhibited same type of potency in scavenging of DPPH and ABTS radicals [28, 29, 30]. So,

the presence of pyrogallol as a polyphenolic compound was a main contributor in potent free radical scavenging activity of WSP of *Ammannia baecifera* leaf.

In XO inhibition assay, WSP and pyrogallol exhibited potent inhibitory effect as compared to allopurinol. Moreover, kinetic studies as described by Michaelis–Menten, were applied to define the modes of inhibition displayed by WSP, pyrogallol and allopurinol against XO. In the presence of WSP, pyrogallol and allopurinol, various concentrations of xanthine were utilized to perform these kinetic analyses of XO inhibition. From these plots, the K_m value for XO was calculated to be 9.25 µmol L^{-1} which was quite similar to the previous report [31]. According to the the Lineweaver–Burk plots of WSP (Fig 5a) and pyrogallol (Fig 5b), there was no intersection found on the y or x axis. Therefore, the Lineweaver–Burk plots revealed that WSP and pyrogallol behaved as mixed-type inhibitors. Previously several XO inhibitors of natural origin like 3,4-dihydroxybenzoic acid and quercetin-3′-glucuronide also exhibited mixed-type mode of inhibition [32, 33].

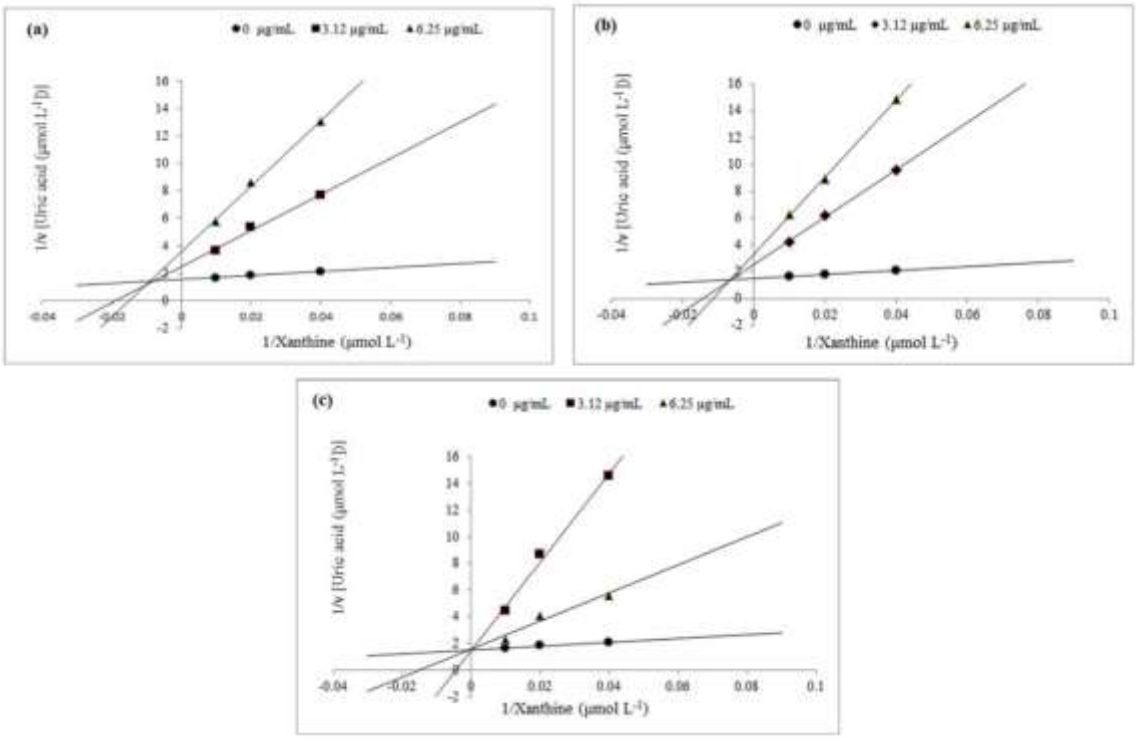

Figure 5: Lineweaver-Burk plots for the inhibition of XO by WSP (a), pyrogallol (b) and allopurinol (c) where xanthine is used as substrate.

As a naturally occurring phenolic compound, the presence of pyrogallol is found in various plants [34, 35]. Previously high reactivity against XO and resulting structural changes were observed for Pyrogallol [31]. So further studies were conducted to clarify its XO-inhibitory mechanism and it was found that under alkaline conditions, chemical conversion of pyrogallol into purpurogallin was happened. This conversion is mainly responsible for the potent XO inhibitory activity of Pyrogallol [31]. So, in this study, alkaline condition (0.1M phosphate buffer; pH 7.6) of XO inhibition assay promoted this type of chemical conversion of pyrogallol that was ultimately responsible for its powerful XO inhibitory activity.

CONCLUSION

The results of this study indicate that pyrogallol plays main role in the XO inhibition and free radical scavenging of *Ammannia baecifera* leaf. So this study gives the scientific clarification for traditional use of *Ammannia baecifera* leaf in the treatment of gout. Moreover, this study also provide scientific basis to intake of pyrogallol in the form of *Ammannia baecifera* leaf for preventing oxidative damages that are caused by free radicals in biological system. As conclusion, it can be suggested that pyrogallol may play an important role in controlling disease and reducing dependence on synthetic chemicals.

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