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Antioxidant, Anti-inflammatory and Xanthine oxidase inhibitory Activity of *Tephrosia purpurea* Plant Extracts

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Abstract

T. purpurea whole plant extract (TPWPE) was evaluated for antioxidant, anti-inflammatory, and xanthine oxidase (XO) inhibitory activities. Antioxidant activity was measured using ABTS and FRAP methods, anti-inflammatory activity was measured by Diene-conjugate and β-glucuronidase assay. *In vitro* XO inhibitory activity was measured by using cow milk xanthine oxidase enzyme. The average antioxidant activity of TPWPE (1-2 µg/mL) in the reacting system revealed significant activity viz; 42.2 (ABTS) and 36.5 (FRAP) percent. The anti-inflammatory activities reveled, 45.40 and 70.50 percent inhibition. The result for XO inhibitory activity by plant extracts reveled, 95.5 % inhibition to that, off control (allopurinol) 92 % inhibition. The kinetic parameters of XO inhibition, revealed noncompetitive mode of inhibition, where, K_m and V_{max} of TPWPE extracts (25 to 100 µg/mL)) were, 0.25 mM/mL and 0.040, 0.036, 0.032 and 0.030 (µg/min) while for positive control K_m and V_{max} is 0.30 mM/mL and 0.045 (µg/min) respectively. Results suggest that, TPWPE can be exploited against diseases associated, with free radical formation and xanthine oxidase activity; further by isolation and structural elucidation of active phytochemicals from TPWPE.

Keywords: *T. purpurea,* Antioxidant, Anti-inflammatory, Xanthine oxidase.

Introduction

Tephrosia purpurea L, (Family: Leguminosae) is a pantropical, polymorphic, branched, perennial herb; found throughout the Indian subcontinent and popularly known as "Sarapunkha" in Sanskrit and 'Purple Tephrosia' in English [1]. The plant used in folk medicine as an antidiabetic, antipyretic, anticancer, and antiulcer agent in addition to its usefulness in treatment of diseases related to oxidative stress, and inflammation [2]. The herb gives a stimulant action on the liver, also act as an appetizer and digestant. *T. purpurea* is very useful in digestive disorders, anorexia, flatulence, abdominal pain, tumors, hemorrhoids, worms, liver and spleen disorders [3, 4]. Free radicals nothing but the reactive oxygen species, which includes hydroxyl, peroxyl, super oxide radicals, hydrogen peroxide, singlet oxygen, and various lipid peroxides. The lipid peroxides are capable of reacting with membrane lipids, proteins, nucleic acids, various metabolic enzymes, and small molecules of living systems. Free radicals play an important role in the initiation and progression of various diseases such as; atherosclerosis, cardiovascular diseases, aging, respiratory diseases, cancer, and gout [5, 6]. Antioxidants are molecules having capacity of preventing or slowing the oxidation of molecules. Oxidation reaction transfers electrons from a substance to an oxidizing agent and produces free radicals thereby causing damage to cells [7]. However most of the cells contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins, and lipids [8]. Antioxidants either remove or prevent these reactive species before they can damage vital cellular components of the cell or tissues [9]. Inflammation is fundamental protective response or a local response to living mammalian tissue injury. This phenomenon develops due to the interaction of the free radicals like ROS and cellular components, resulting in cellular damage, and tissue injury [10]. Gout develops due to the deposition of uric acid in the form of urate monohydrate crystals in the synovial joints during purine catabolism by xanthine oxidase (XO) [11]. XO catalyses the conversion of hypoxanthine to xanthine and xanthine to uric acid with concomitant production of hydrogen

peroxides and superoxide anions as byproducts; which leads to the oxidative stress in cell and development of gout in human beings [12]. Recently, the most important reported biological properties of secondary metabolites are due to their electron transfer capacity, free radical scavenging, chelating abilities, oxidase inhibitors, and anti-inflammatory activities [13]. The previous researcher reported that, the plant contains flavonoids, chalcones, and alkaloids as bioactive constitutes [14]. Depending up on this facts, the study is aimed to evaluate the effect of TPWPE as an antioxidant, anti-inflammatory, and xanthine oxidase inhibitor, which is mainly involved in formation of uric acid; leading to free radical induced damage and gout.

Materials and methods

Plant material and preparation of *T. purpurea* whole plant extract (TPWPE)

T. purpurea plant was collected in August-2011 from local forest of Nanded, India. The plant taxonomically identified and deposited in department (Voucher No SRT/BT/P/SN/ 101). The whole plant were shade dried for a week, grounded by using mortar and pestle and the powder was extracted with methanol (90%) by using Soxhlet apparatus for 4 h, then filtered, concentrated under reduced pressure at 60 ^{0}C in a vacuum rotator evaporator to dryness and used for activity measurements as per the need [15].

Chemicals

2, 2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid diammonium salt (ABTS), 2, 4, 6-tripyridyl-s-triazine (TPTZ), fluorescein dye, trolox, quercetin, EDTA, Folin-ciocalteu reagent, ferric chloride, acetylsalicylic acid, methanol, β-glucuronidase, Xanthine oxidase, allopurinol, xanthine were purchased from Hi-Media Laboratories Ltd. Mumbai, India and Sigma Aldrich Chemicals, Co, USA. All chemicals were of AR grade and used without further purification unless stated otherwise. The hen's eggs were purchased from local market.

ABTS radical scavenging activity

ABTS 19 mg (7 mM) was oxidized with potassium persulfate 3.3 mg (2.45 mM) overnight in the dark bottle for about overnight in dark. The working solution was then diluted with ethanol to an absorbance of 0.75 at 734 nm. A standard calibration curve was constructed for trolox at 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 2 mM. An aliquot (10 µl) of *T. purpurea* whole plant extracts (1, 1.5 and 2 µg/mL conc.) was mixed with 1.0 mL of ABTS radical cation working solution in cuvette and absorbance was read at 734 nm after 30 min. The activity was expressed as the effective concentration of drug necessary to give a 50 % reduction in the original absorbance [16].

Ferric reducing antioxidant power (FRAP) Assay

The stock solutions were prepared by using 300 mM acetate buffer (3.5 g sodium acetate trihydrate and 20 mL acetic acid, pH 3.6). The reagent was prepared by using TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3.6H_2O$ so as to form the FRAP solution. The working solution (FRAP) was warmed at 37 ^{0}C before use. *T. purpurea* whole plant extracts with different concentrations (1, 1.5 and 2 µg/mL) allowed to react with 28.50 µl of the FRAP solution for 30 min in dark condition. Absorbance of the colored product (ferrous tripyridyltriazine complex) was recorded at 595 nm. Trolox was used as standard and the standard curve was linear between 25 and 800 µM of torolox. The results were expressed as µM torolox equivalent (TE)/g fresh mass. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve [17].

Conjugated diene assay

Conjugated diene assay involves the preparation of RBC cell membrane. The blood samples collected with addition of EDTA (2 mg/mL) as an anticoagulant; then it was centrifuged and the plasma was aspirated. The blood cells were washed three times with saline (0.89%) to 0.5 mL of cells, 7 mL of ice-cold distilled water was added and left overnight at 0 ^{0}C, the hemolysate was separated by centrifugations in cooling centrifuge at 10,000 rpm for 20 min. The pellet was washed twice with distilled water, followed by centrifugation for 10 min and then suspended to a 10 mL of Tris-HCl buffer (0.1 M, pH 7.4) and the resultant solution was then used as a membrane solution. The membrane solution (1.0 mL) was mixed with 5 mL of chloroform: methanol (2:1) and 2 g of *T. purpurea* whole plant extracts. The mixture was centrifuged at 10,000 RPM for 15 min to separate the two phases. The chloroform layer was removed by using separating funnel and dried at 45 ^{0}C in a water bath. The lipid residue was dissolved in 1.5 mL of cyclohexane and hydro peroxides generated were detected at 233 nm spectrophotometrically against a cyclohexane as blank. Acetylsalicylic acid (1mM) was used as a standard drug. The percent activity in all the parameters was calculated by using the standard formula [18, 19].

% Activity = (1-T/C) X 100, where T and C = absorbance of test and control samples respectively.

β-Glucuronidase inhibition assay

For this assay the 2.5 mM p-nitrophenyl-β-D-glucopyranosiduronic acid was incubated with 1 mg (0.1 mL) of *T. purpurea* plant extract in acetate buffer (0.1M, pH 7.4) for 5 min followed by addition of 0.1 mL of β-glucuronidase solution. The mixture was further incubated for 30 min followed by addition of 2 mL NaOH (0.5 N) for termination of the reaction. The amount of reaction product formed was observed and absorbance was recorded spectrophotometrically at 410 nm. The salicylic acid (1 mM) was used as reference drug for comparative study [20].

Xanthine oxidase inhibitory activity

The inhibitory activity of *T. purpurea* plant extract was determined using the standard inhibitor allopurinol (5 mM), which was then diluted with phosphate buffer (0.1 mM) to obtain 5, 10, 15, 20 and 25 µM solutions. *T. purpurea* whole plant extract was dissolved in 1 mM phosphate buffer (pH 7.5) to make concentrations 25, 50, 75 and 100 µg/mL, The inhibitory activity of *T. purpurea* whole plant extract was determined using a slight modification of reference methods [21, 22]. Briefly as; Control: 7.0 µL of XO buffer solution (0.4U/ mL) were added to 0.1 M phosphate buffer pH 7.8 (130.0 µL). The reaction mixture was then incubated at 37 ^{0}C for 10 min. The 70.0 µL of 40 µM xanthine buffer solution were added to the mixture and the absorbance was recorded at 295 nm spectrophotometrically at 37^{0}C for 10 min. The blank solution contained 7 µL of phosphate buffer solution. The test was performed in triplicate. Sample test: 7.0 µL of XO buffer solution (0.4U/ mL) were added to a solution consisting of 0.1 M phosphate buffer pH 7.8 (80.0 µL) and different concentrations of *T. purpurea* whole plant extracts (25.0, 50.0, 75.0 and 100 µL) were treated in the same manner as the control. 4.0 µL of phosphate buffer solution were used instead of XO solution (0.4 U) for blank tests. The test was performed in triplicate. Enzyme inhibitory activity was determined by quantifying the amount of uric acid formation from xanthine in the reaction mixture. The assay mixture contained both *T. purpurea* plant extract 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 for the reaction. XO activity was expressed as % inhibition of XO, calculated as (1-B/A) X100, where A is the change in absorbance of the assay without the *T. purpurea* whole plant extract samples. (∆ Abs with enzyme - ∆ Abs without enzyme), and B is the change in absorbance of the assay with the *T. purpurea* plant extracts (∆ Abs with enzyme - ∆ Abs without enzyme). The enzyme kinetics was similar to XO assay method, which was expressed in terms of V_{max} and K_m.

Results & Discussion

Antioxidant activity

The results for ABTS and FRAP radical scavenging (% activity) of *T. purpurea* plant extract summarized in Table 1. The *T. purpurea* plant extract under study was effective towards the scavenging of ABTS and FRAPS radicals. The overall range of ABTS and FRAP scavenging activity of *T. purpurea* plant extract was 44.86 and 38.80 percent respectively as compared to the reference compound trolox was, 40.80 and 32.25 respectively. The average activity with Antioxidant capacities of plant extracts not only depend on plant composition, but also on the conditions of the test used. There are numerous published methods measuring total antioxidant capacity *in vitro*, which can be classified into two types: assays based on hydrogen atom transfer (HAT), and assays based on electron transfer (ET). HAT-based assays, like the ORAC assay, apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals [23, 24]. ET-based assays measure the capacity of an antioxidant to reduce an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant concentration [25]. No single method is sufficient; more than one type of antioxidant capacity measurement needs to be performed to take into account the various modes of action of antioxidants [26]. In this study, we determined the free radical scavenging capacities of the *T. purpurea* plant extracts using ABTS, and their ferric reducing capacities using the FRAP assay. ABTS, and FRAP assays have been widely used to determine the antioxidant capacities of plant extracts as they require relatively standard equipment and deliver fast and reproducible results. Indeed, an inter laboratory comparison of antioxidant methods for measuring antioxidant potential published recently showed that ABTS assays are the easiest to implement and yield the most reproducible results [27]. The ABTS and FRAP assays gave comparable results for the antioxidant activity measured in *T. purpurea* whole plant extract.

Anti-inflammatory activity

Anti-inflammatory activity was determined by using diene-conjugate and β-glucuronidase assays (Table 2). *T. purpurea* plant extracts revealed excellent anti-inflammatory activity profile showing 58.2 and 68.4 % inhibitions respectively. The inhibition percent for control was 50 and 72 % respectively. The results presented for *T. purpurea* whole plant extracts revealed excellent anti-inflammatory activity. It is known that during inflammation and associated processes, there is an increased production of superoxide ions. It may be possible that the inhibition of superoxide generation in peritoneal macrophages is related to the anti-inflammatory activity of *T. purpurea* [28]. The β-glucuronidase mainly occurs in lysosomes of neutrophils and play important role as mediators in initiation and progression of inflammation [29]. The hydroperoxides (diene-conjugates) generation is one of the intermediate steps in membrane lipid peroxidation [30]. The lipid peroxidation phenomenon plays a vital role in many inflammatory disorders. The lipid peroxidation results in oxidative modifications of the apoprotein which is mainly involved in macrophage uptake and atherogenesis [31]. The result indicates that the *T. purpurea* whole plant extract may reduce the lipid peroxidation by virtue of their antioxidant and anti-inflammatory activity. HET-CAM assay is a novel method for anti-inflammatory assay. The inflammatory condition was induced by sodium dodecyl sulfate (50 µg/pellet) and was observed after 24 hours. The phenyl butazone (control) has 75 to 80 % inhibition as a standard. However, the *T. purpurea* plant extract revealed very weak % inhibition by this assay.

Table. 1 Antioxidant activity of methanolic extract of *T. purpurea*

	% antioxidant activity (µM TE or QE/g FM)	
Sample	ABTS	FRAP
1 µg/mL	38.90± 5.3	34.90± 2.6
1.5 µg/mL	42.84± 4.2	35.80± 5.1
2 µg/mL	44.86± 7.2	38.80± 4.9
Trolox	40.80± 3.8	32.25± 2.4
[Values represent Mean ± S.E from three experiments]		

Table. 2 Anti-Inflammatory activities of methanolic extract of *T. purpurea*

Sample	% Inhibition	
	DC	βG
1 µg/mL	42.8± 5.6	58.3± 6.3
1.5 µg/mL	45.2± 5.2	61.0± 5.4
2 µg/mL	58.2± 3.5	68.4± 7.2
Phenyl butazone	50.0± 2.7	72.0± 4.3

Table. 3 Xanthine oxidase (XO) inhibitory activity of *T. purpurea* extracts

	Xanthine oxidase Inhibition (%)			
Test Samples	**25 µg/mL**	**50 µg/mL**	**75 µg/mL**	**100 µg/mL**
T. purpurea extract	35.2± 1.8	50.1± 3.5	76.5± 4.1	90.5± 3.2
Control	**5 µg**	**10 µg**	**15 µg**	**20 µg**
Allopurinol	40.0± 2.6	65.2± 1.8	85.0± 1.6	97.0± 2.4
[Values represent Mean ± S.E from three experiments]				

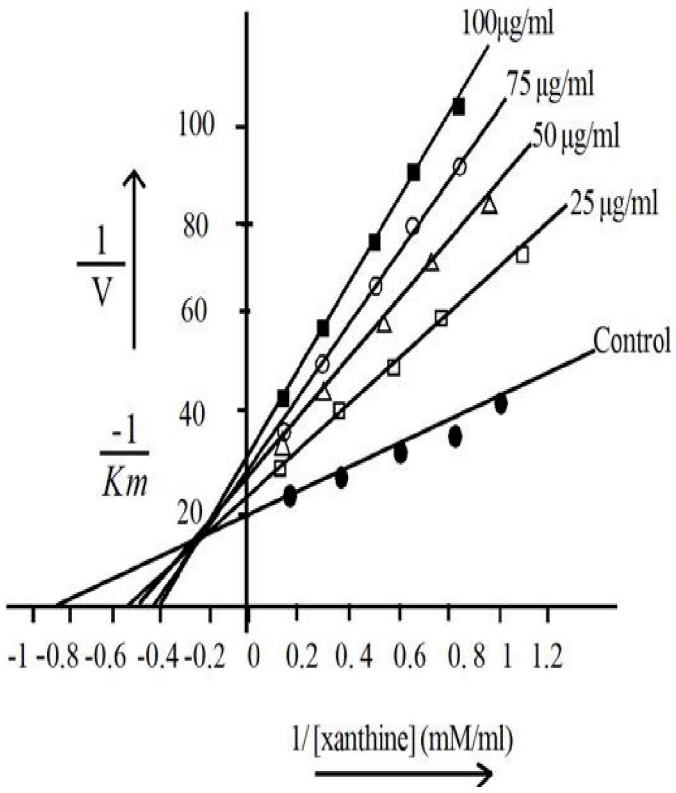

Figure1. lineweaver-burk plots of inhibition of xo(xanthine oxidase) in the presence of tephrosia purpurea(□-Δ-○-■) with different concentration and allopurinol in absence(●), xanthine as the substrate

XO inhibitory activity

T. purpurea whole plant extracts demonstrated dose dependent XO inhibitory activity at 25, 50, 75 and 100 µg/mL (Table 3). The minimal inhibitory activity was 35.2 % at 25 µg/mL and maximum 90.5 % at 100 µg/mL which was approximately similar to that of standard drug allopurinol (97 %) at 20 µM/mL. The inhibition of XO results in a decreased production of uric acid, measured spectrophotometrically at 295 nm.

The significant inhibition of XO by extract might suppress the production of active oxygen species or uric acid *in vivo* under the conditions that XO works. The inhibition percentage by *T. purpurea* plant was comparable to that of allopurinol (20 µg /mL), a therapeutic drug used to treat gout. The kinetic analysis using Lineweaver Burk plot revealed that, the *T. purpurea* plant extract displayed high inhibitory activity. The pattern of inhibition is non competitive type of inhibition in presence of *T. purpurea* where in V_{max} is decreased and K_m appears to be unaltered with respect to Xanthine as substrate (Fig. 1). It indicates that, the binding of extract may occur with the free enzyme or the enzyme substrate complex. The significant inhibition of XO by *T. purpurea* plant extract may suppress the production of reactive oxygen species or uric acid *in vivo* under the conditions that Xanthine oxidase works. The inhibition percentage by *T. purpurea* plant extract is comparable to that of allopurinol, a therapeutic drug used to treat gout. From this study it was concluded that, *T. purpurea* whole plant extract exhibited the strong antioxidant and anti-inflammatory activity, which is might be due to the phytochemicals present in this plant, which may have great potential to acts as free radical scavenger to reduce the oxidative stress and inflammation and might prevent the diseases caused due to over production of reactive oxygen species or free radicals in living beings. Further, the bioguided fractionation of the active phytochemicals from *T. purpurea* plant extract is being carried out, by aiming the formulation of a safer and efficient drug to prevent the oxidative stress and related diseases. *T. purpurea* plant extract revealed potent XO inhibition activity. It indicates that the phytochemicals present in this plant extract may also be useful for the treatment of gout and hyperuricemia, which are correlates with the ethno botanical data on the use of these plants in Indian folklore and ayurveda. The data investigated in this study provides the basis for further investigation on this plant to isolate the active constituents and drug developments against the disease related to oxidative stress and inflammation

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