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PHYTOCHEMICAL STUDIES, CHARACTERISATION OF NOVEL FLAVONOL ISOLATE OF *ZANTHOXYLUM TETRASPERMUM* WOODHEART AND REPORT ON ITS ANTIOXIDANT ACTIVITES

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Abstract

Kaempferol 5, 7-di-O-methyl-6-prenyl-4'-O-glucoside was isolated from *Zanthoxylum tetraspermum* woodheart. The estimation like phenolic, flavonoid, tannin, flavonol contents of crude woodheart extract proves the potent antioxidant characteristic of the species. Diethyl ether (Et_2O) extract has exhibited significant effect for all these estimations as compared to ethyl acetate (EtOAc) and light petrol extracts. Significant contents for phenolic, flavonoid, tannin and flavonol contents in Et_2O extract were 75.89, 115.39, 144.14 and 61.65 mg respectively. *In vitro* assays like 1,1-diphenyl-2-picrylhydrazyl (DPPH) - radical scavenging and reducing power activity have exhibited the extraordinary potential of the Et_2O, EtOAc crude extracts and flavonol 4'-O- glucoside. The generated data has provided the basis for therapeutic value of the species.

Keywords: *Zanthoxylum tetraspermum*, Woodheart, Kaempferol, DPPH radical scavenging, reducing power.

Introduction

In continuation of our studies on phytochemical constituents of Indian medicinal plants, green synthesis of nano particles and biological studies[1-7] we now set our objective to study antioxidant efficacy, to screen and estimate phytochemicals like phenols, flavonoids, tannin and flavonol and to isolate and characterise the possible active flavonol constituent of woodheart part of *Zanthoxylum tetraspermum* belonging to Rutaceae family. The species has antirheumatic, antispasmodic, diuretic, odontalgic activities. The bark and roots of the plant along with the fruit are diaphoretic, stimulant and a useful tonic in debilitated conditions of the stomach and digestive organs. The stem bark of

the species have shown the presence of secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, sterols, saponins, tannins in higher levels in hydroethanolic and aqueous extracts and some minerals in the plant extract[8]. Two benzophenanthrene alkaloids, 8-acetonyldihydronitidine and 8-acetonyldihydrovicine were isolated from the stem bark along with liriodenine, sesamin, lichexanthrone and (+)piperitol-γ, γ-dimethyl allyl ether[9]. A literature search revealed that there are no reported chemical and biological studies on woodheart of the plant.

Materials and methods

Chemicals

Gallic acid, Tannic acid, (±) Catechin, Glucose, Ascorbic acid, α-Tocopherol, Folin-Ciocalteu's reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH) used were of analytical grade purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant collection and extraction

Healthy *Z.tetraspermum* woodhearts were collected from Kolli Hills, Western Ghats, Namakkal District, Tamil Nadu, India identified and authenticated by Taxonomist, Kandaswami Kandar's College, Velur, Namakkal district, Tamil Nadu, India.

The chiped woodhearts were washed with tap water followed by distilled water, shade dried and powdered. The powdered woodhearts were subjected to successive extraction with light petrol (60-80 ºC), peroxide free Et_2O and EtOAc using soxhlet extractor. The extracts were dried in vacuum pump at 40ºC. A part of the residue from EtOAc fraction was taken up in acetone (Me_2CO) and left in an ice chest for two days when a pale yellow solid separated. It came out as pale yellow plates (compound 1) on recrystallisation from hot water. The dried crude extracts of light petrol, Et_2O and second part of EtOAc were stored in freezer at 0ºC. Light petrol and Et_2O extracts did not yield any isolable solid.

Phytochemical screening

The preliminary phytochemical screening tests were carried out to identify the useful constituents by standard methods[10].

Determination of total phenolic contents

The total phenolics in the light petrol, Et_2O and EtOAc crude extracts were estimated by spectrophotometric assay[11]. One milliliter of sample (concentration 1 mg/mL) was mixed with 1mL of Folin and Ciocalteu's phenol reagent. After

3 min, 1mL of saturated sodium carbonate solution was added to the mixture and made upto 10mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used for constructing the standard curve (20-100 µg/mL, Y=0.025x + 0.0378, R^2=0.997) and the results were expressed as µg of gallic acid equivalents/mg of extract (GAEs).

Determination of total flavonoid contents

Flavonoid contents in the light petrol, Et_2O and EtOAc crude extracts were determined by spectrophotometric method[11] 250µL extract (concentration 1mg/mL) was mixed with 1.25mL of distilled water and 75 µL of a 5% $NaNO_2$ solution. After 5 min, 150µL of 10% $AlCl_3$ solution was added. After 6 min, 500 µL of 1 M NaOH and 275 µL of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. (±)-Catechin was used to calculate the standard curve (20-160 µg/mL, Y= 0.0021x + 0.0876, R^2 = 0.9886) and the results were expressed as µg of (±)-Catechin equivalents (CEs) per mg of extract.

Estimation of total flavonol contents

One milliliter of sample extract (concentration 1 mg/mL) was mixed with 1 mL aluminium trichloride (5 mg/mL) and 3 mL sodium acetate (25mg/mL). The absorbance at 440nm was read after 2.5 h. The absorption of standard rutin solution (0.5mg/mL) in methanol was measured under the same conditions[12]. All determinations were carried out in duplicates. The amount of flavonols in plant extracts in rutin equivalents (RE) were calculated by the following formula. $X = (A.m_o)/(A_o.m)$, where X is the flavonol content, mg/mg plant extract in RE, A is the absorption of plant extract solution, A_o is the absorption of standard rutin solution, m is the weight of plant extract (mg), and m_o is the weight of rutin in the solution (mg).

Estimation of total tannin contents

Tannin content of the light petrol, Et_2O and EtOAc crude extracts was measured by Folin-Denis method[13]. The various extracts (50 µL) were made up to 7.5mL by adding double distilled water. Then 0.5mL Folin-Denis reagent and 1 mL of Na_2CO_3 were mixed with it. Again the volume was made up to 10 mL by double distilled water. Absorption was recorded at 700 nm. Tannic acid was used to calculate the standard curve (20-120 µg/mL, Y = 0.0008x + 0.0144, R^2=0.9893) and results were expressed as µg of tannic acid equivalents per mg of extract.

Characterization of compound-1

Compound-1 was characterized by Paper Chromatography (PC), UV, ^{1}H and ^{13}C NMR and FTIR studies.

DPPH radical scavenging activity: Various concentrations of light petrol, Et_2O and EtOAc crude extracts and compound 1 of *Z. tetraspermum* woodhearts in methanol (0.3mL) were mixed with 2.7mL of methanol solution containing DPPH radicals (6×10^{-5}mol/L). The mixture was shaken vigorously and allowed to stand for 60 min in the dark. The reduction of the DPPH radical was determined by reading the absorbance at 517 nm[14]. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration, using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract is added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radical-scavenging activity (IC_{50}) was calculated from the graph of RSA percentage against extract concentration. Ascorbic acid and α-tocopherol were used as standards for crude extracts. Ascorbic acid and butylated hydroxyl anisole (BHA) were used as standards for compound-1.

Reducing power

The reducing power of light petrol, Et_2O and EtOAc crude extracts and compound 1 of Z.*tetraspermum* woodhearts extracts was determined[13]. Various concentrations of light petrol, Et_2O and EtOAc crude extracts and compound 1 in methanol (1mL), phosphate buffer (1mL, 0.2 M, pH = 6.6) and potassium ferricyanide (1mL, 10 mg/mL) were mixed together and incubated at 50ºC for 20 min. Trichloroacetic acid (1 mL, 100 mg/mL) was added to the mixture and centrifuged at 8,000 rpm for 5 min. The supernatant (1mL) was mixed with distilled water (1mL) and ferric chloride (0.1mL, 1 mg/mL) and then the absorbance was measured at 700 nm.

Statistical analysis

The results are expressed as mean values and standard deviation (SD), n=3. Data were analysed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test using SPSS software. Values of $P < 0.05$ were considered statistically significant.

Results

Phytochemical screening: The phytochemical screening of *Z.tetraspermum* woodheart in various extracts evidence their antioxidant properties by revealing the presence of numerous secondary metabolites. The presence of them from copious, slightly to moderately present in all extracts has been listed in Table-1.

Table-1: Phytochemical screening of *Z. tetraspermum* woodheart in various extracts.

S.No	Phytochemical Constituents	Various Extracts		
		Light Petrol	Diethyl ether	Ethyl acetate
1.	Alkaloids	+	+	++
2.	Flavonoids	+	+++	+++
3.	Flavones	-	++	++
4.	Flavanones	-	+	-
5.	Flavonols	+	+++	++
6.	Isoflavones	-	-	-
7.	Glycosides	-	+	+
8.	Tannins	+	+++	+++
9.	Phenolics	+	+++	+++
10.	Amino acids	-	+	-
11.	Steroids	-	-	+
12.	Proteins	-	+	+
13.	Carbohydrates	-	+++	++
14.	Saponins	+++	-	+
15.	Terpenoids	-	++	++
16.	Fats/Oils	+++	-	-
17.	Anthocyanins	-	-	-
18.	Leucoanthocyannins	-	-	-

+++ = Copiously present, ++ = Moderately present, + = Slightly present, - = Absent.

Total phenolic content

The Et_2O extract recorded the highest content of about 75.89 µg/mg. The next highest was observed in EtOAc extract and the least was observed in light petrol extract of about 13.84 µg/mg.

Total flavonoid content

The Et_2O extract recorded the highest content of flavonoid too (115.39 µg/mg) followed by EtOAc extract (49.98 µg/mg) with the least value for light petrol extract (10.70 µg/mg).

Total flavonol content

The trend continued with Et_2O extract exhibiting maximum (61.65 µg/mg) followed by EtOAc extract (42.86 µg/mg) and light petrol extract the minimum (20.87 µg/mg).

Total tannin content

The same trend continued. Et_2O extract recorded the highest value of 144.14 µg/mg. EtOAc extract recorded intermediate value of 74.68 µg/mg. Light petrol recorded the lowest value of 14.42 µg/mg.

Table-2: Estimation of various contents of *Zanthoxylum tetraspermum* woodheart in various solvents.

Phytochemical constituents (µg/mg extract)	Ethylacetate	Diethylether	Light petrol
Total phenolic content*	66.75 ± 1.19	75.89 ± 0.68	13.84 ± 0.98
Total flavonoid content*	49.98 ± 0.49	115.39 ± 1.61	10.70 ± 0.49
Total flavonol content*	42.86 ± 0.001	61.65 ± 0.001	20.87 ± 0.001
Total Tannin content*	74.682 ± 0.572	144.143 ± 1.334	14.421 ± 0.143

*The values are means of three replicates with standard deviations (mean ± S.D.; n=3), $p < 0.05$

Characterization of compound 1

Compound 1 crystallized from EtOAc fraction and recrystallized from Me_2CO, hot water as pale yellow plates (1g), m.p. 178-80° (Found: C, 61.50, H, 6.02; $C_{28}H_{11}O_{32}$ requires C, 61.76, H, 5.88); UV(MeOH): 266, 285, 323; +NaOMe: 262, 282; +$AlCl_3$: 276,285, 327; + $AlCl_3$ + HCl : 276, 282, 326; + NaOAc: 281, 322; + NaOAc + H_3BO_3: 278, 282, 324. ^{1}H NMR (400MHz, Cd_3Od) δ 6.80 (1H, s, H-6), 7.00 (2H, d, J= 8.5Hz, H-3' and H-5') 7.60 (2H, d, J=8.5Hz, H -2' and H-6', 5.0 (1H, d, J=7Hz, H -1"). The vinylic methine proton of phenyl residue appeared at δ 5.40 ppm (t, J=4Hz). The resonance of allylic methylene protons appeared at1.80ppm. Two methyl groups of prenyl residue appear at δ 1.20 and 1.40 ppm respectively. ^{13}C NMR (75.43 MHz, DMSO – d_6): 148.01(C-2), 139.76 (C-3), 174.00 (C-4), 160.30 (C-5), 111.82 (C-6), 161.94 (C-7), 95.43 (C-8), 154.29 (C-9), 106.62 (C-10), 119.17 (C-1'), 129.00 (C-2'), 114.82 (C-3'), 155.99 (C-4'), 114.82 (C-5'), 129.00 (C-6'), 100.65 (C-1"), 73.80 (C-2"), 76.81 (C-3"), 70.31 (C-4"), 75.69 (C-5"), 63.11 (C-6"), 55.69 (5-OCH_3), 56.40 (7-O-CH_3), 20.19 (C-1'"), 25.26(C-2'"), 25.78 (C-3'"), 17.87 (C-4'" and C-5'"). IR (ν_{max}) Cm^{-1} 3372, 2925, 2858, 1713, 1454, 1274, 825.

DPPH radical scavenging activity

Variation in the concentration of DPPH free radical with various concentrations of light petrol, Et_2O and EtOAc crude extracts and compound 1 are depicted in Fig 1 & Fig 2. IC_{50} values represent the concentration of an inhibitor that is required for 50% inhibition of its target i.e. DPPH radicals.

Order of scavenging effects of extracts based on their lower IC_{50} values

a) Ascorbic acid > α-tocopherol > Et_2O extract > EtOAc extract > light petrol extract

b) BHA > Ascorbic acid > Compound 1

Figure-1: DPPH radical scavenging activity of *Z. tetraspermum* woodheart in various extracts.

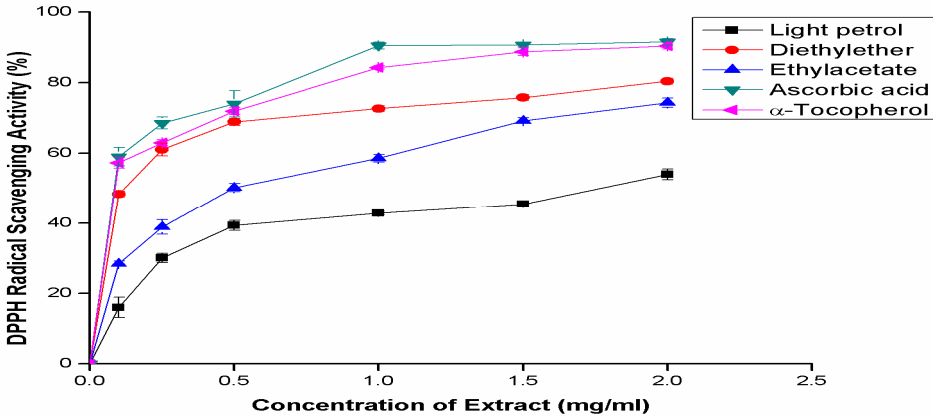
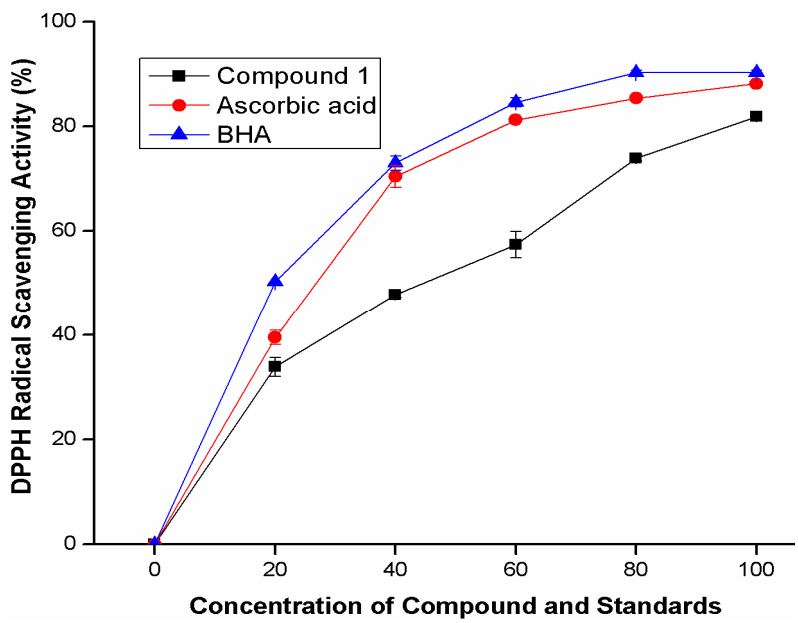


Figure-2: DPPH radical scavenging activity of compound 1 and standards.



Reducing power

Increase in reducing power of extract with increase in concentration is shown in Fig 3 & Fig 4. The reducing power of extract based in their lower EC_{50} values which represent the concentration of extracts that provided 0.5 absorbance at 700 nm, is of the same order as DPPH scavenging activity.

Figure.3: Reducing power activity of *Z. tetraspermum* woodheart in various extracts.

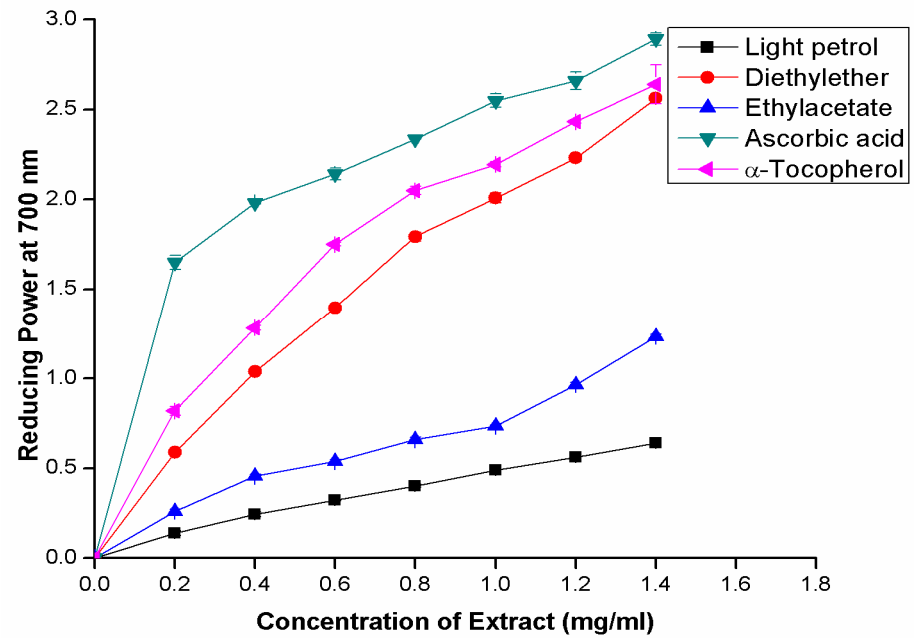
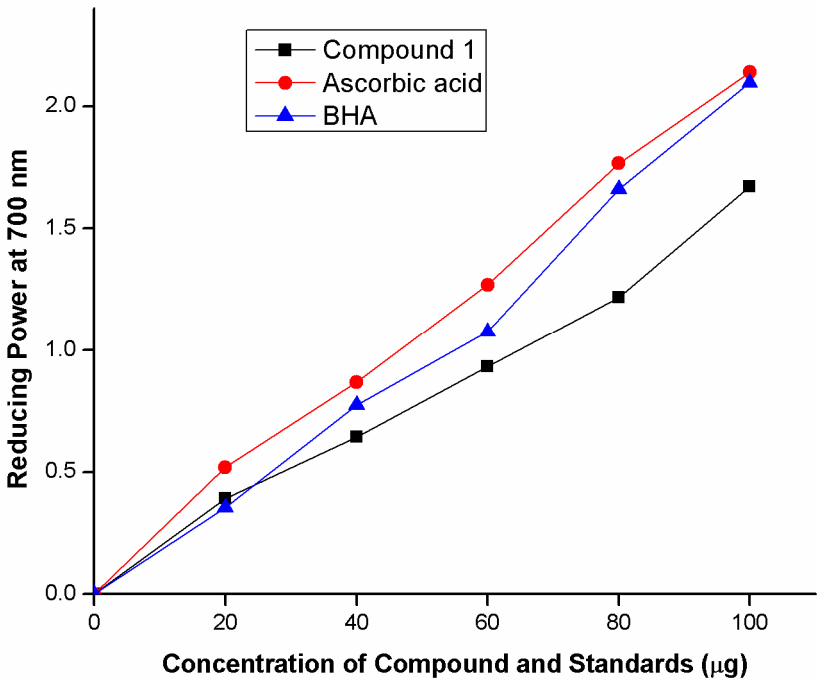


Figure.4: Reducing power activity of compound 1 and standards.



Discussion

UV and IR spectra and a positive Molisch test indicated compound 1 to be a flavonol glycoside with substitution at 4' – hydroxyl[15]. Hydrolysis of compound 1 with mineral acid gave its aglycone and D-glucose. The β linkage of the glucose moiety to 4'-hydroxyl group was evident from the large coupling constant of H-1" signal in ^{1}H NMR of compound 1[16]. The upfield shift by 3.21 ppm of C-4' in ^{13}C NMR of compound 1 as compared to the signals of authentic kaempferol (δ 159.20) further supported glycosylation at C-4'[17]. The prenyl moiety at C-6 and methoxy groups at C-5 and C-7 exhibited due resonances in ^{13}C – NMR. On the basis of the above, compound 1 was characterised as 6-prenyl -5, 7-di-O-methyl Kaempferol 4'-O- glycoside. The hydrolysed aglycone of compound 1 was characterised as 6-prenyl -5, 7-di-O-methyl Kaempferol.

Active compounds present in plants facilitate them to possess antioxidant activity. The viability of the used methods in determining phenolic, flavonoid, flavonol and tannin contents in EtOAc, Et_2O and light petrol extracts is proved by the results. The data unambigously indicate the richest source of phenolics, flavonoids, flavonols and tannins in Et_2O extract. Equally EtOAc extract had showed significant contents. The contents constitute the main class of natural

antioxidants in plants. Tannins are polyphenolic compounds that have direct antioxidant activity, especially the scavenging properties.

DPPH radical scavenging activity and reducing power activity are considered reliable method to quantify antioxidant activities. Highest scavenging activity was observed with Et_2O extract whose IC_{50} value was (0.12 mg) as opposed to IC_{50} value of ascorbic acid (0.08 mg) and α-tocopherol (0.08 mg) the well known antioxidants. Compound 1 exhibited IC_{50} value 43.95µg/mg as against ascorbic acid 26.68µg/mg and BHA 20.07µg/mg. It is appreciated that the scavenging activities of Et_2O crude extract and compound 1 are nearer the standards. So, the flavonol glycoside, 6-prenyl -5, 7-d-O-methyl kaempferol can prove its therapeutic action in preventing or slowing the oxidative stress related degenerative diseases. The highest IC_{50} value is given by light petrol (1.75 µg) indicating its least efficiency. Scavenging of DPPH radical was observed to increase with increasing concentration of extracts. It is shown in Fig 1 & 2.

Redox properties of phenolics and flavonoids facilitate them to absorb and neutralize free radicals, quench singlet or triplet oxygen or decompose peroxides thereby cause its antioxidant activity. Tannins are the polyphenolic compounds that have direct antioxidant activity, especially the scavenging properties[18]. The reducing power of all extracts increased with increase in their concentration (Fig 3&4). EC_{50} for Et_2O crude extract and compound 1 are 171 and 27.47 µg against their respective standards.

Conclusions:

The result of the present phytochemical studies are valuable as it has established that the novel flavonol glycoside 6-prenyl-5, 7-di-O-methyl kaempferol 4'-O-glucoside isolated from woodheart Z.*tetraspermum* is highly responsible for its antioxidant activity. Moreover the studies reveal the content of various phytochemicals. Further studies to elucidate antioxidant mechanism of the isolate and the existence of possible synergism and their effects through *in vivo* studies are needed to evaluate their natural biological function.

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