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Antioxidant and Anti-Cholinergic Activities of Phenolic Compounds Isolated From *Thymus Linearis* Collected from Dir, Pakistan

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Abstract

Due to the presence of polyphenol with strong natural antioxidant activity, thyme has been considered as a dietary supplement for many centuries. The main goal of the study was carried out to explore the phenolic compounds from *Thymus linearis* collected form Dir, Pakistan along with its antioxidant activity. From the methanolic extract of *Thymus linearis*, six compounds were isolated and identified as p-cymene, oleanolic acid, luteolin (LN), 4,5 dicaffeoyl quinic acid (DCQA), rosmarinic acid (RA) and salvianolic acid (SA) by column chromatography method. Antioxidant activity of isolated compounds by DPPH method, superoxide radical scavenging and ABTS assay as well as anticholinergic activity were measured with different concentrations which showed the results significantly. Data obtained from results of antioxidant and anticholinergic assays showed that IC50 values different doses were in order as DCQA>SA>RA>LN. So the study reveals that *Thymus linearis* contained phenolic compounds having strong natural antioxidant activity even at low dose ($p<0.05$). So it can be suggested that by adding the thymus in our daily life food, oxidative stress related disease can be combated due to its strong antioxidant activity.

Keywords

Luteolin; Rosmarinic acid; Salvianolic acid; Dicaffeoyl quinic acid; Antioxidant

Introduction

Plants derived medicines have been used against life threatening disease since ancient times and their demands have been increasing. Although many medicinal plants have been documented for various activities but others are yet to be verified. In recent years studies on antioxidants that consumption of natural antioxidants play a vital role in reduction of occurring of diseases due to oxidative damage at cellular level. Therefore, consideration has been focused mainly in finding the natural antioxidants for preventing the human suffering from such diseases and get improvements in their health [1,2].

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In English and Persian, thymus genus having commonly is known as thyme and azore/avishan and it is related to family Lamiaceae [3,4]. Thymus genus comprises of about 928 species including sherbs, herbs, perineal and herbaceous [4]. Because of carminative, antiseptic, anti-inflammatory, antispasmodics properties, it has been a common part of traditional herbal medicine. Antifungal, antibacterial, antioxidant activities had been shown by many species of thymus [4,5]. Among various types of phytconstituents, phenolic compounds have particular attention and act as natural antioxidants. Studies showed that genus thymus is a rich source of phenolic constituents having strong antioxidant properties. [6]. *Thymus linearis*, one of the species of Thyme genus, is distributed widely in Himalyas. In India, *T. linearis* has been widely spread in Kumaun Himalaya of Uttarakhand. General area of distribution includes North India, Kashmir, Nepal, Tajikistan, Japan and Iran, Pakistan and Afghanistan [7-9]. Anti-herpes viral activity of *Thymus linearis* has been reported scientifically [10,11] and abdominal disturbance, etc [12]. Significant activity against ampicillin-resistant *Escherichia coli* has been shown by its essential oil containing thymol and carvacol but exhibited poor anti-haembiocrystallization activity. Furthermore, anticancer activity against MCF-7, LNCaP and NIH-3T3 cell lines andante bacterial activity was also shown by thyme oil [13,14]. Due to presence of phenolic compounds in different species of thymus such as cinnamic acid, ferulic acid, isoferulic acid (IFA), chlrorgenic acid, thymus has been well known for the treatment of various neurodegenerative diseases. Chemical constituent investigation has been done in the essential oil of thymus linearis but there was no reporting about the presence of phenolic compounds from leaves extract. It has been known that phenolic compounds are known to exhibit many pharmacological effects such as antioxidant; anti-inflammatory [15]. But yet there is no report about the isolation and presence of phenolic compounds in *Thymus linearis* methanolic extract. So this is the first time reporting about presence of phenolic compounds in *Thymus linearis*. Therefore, the present study is to isolate the phenolic compounds from methanolic extract of TL along with its pharmacological activity such as antioxidant and Acetyl cholinesterase activity.

Materials and Methods

Apparatus and chemicals used

Solvents used in experiments were of analytical grade, n-hexane (Hx), dichloromethane (DCM), ethyl acetate (EA), n.Butanol (n-BuOH) and methanol as well as silica gel 60 (230-400 mesh) and sephadex were obtained from MERCK.1,1 – diphenyl-2-picrylhydrazyl (DPPH), Tris HCL, Ascorbic acid and Trolox were obtained from Sigma Aldrich chemicals. Optizen 2120 spectrophotometer were used to measure UV spectra. High performance liquid chromatography (HPLC) was used. Formic acid, methanol and other solvents were used of HPLC grade in HPLC analysis. Nuclear magnetic resonance (NMR) was recorded on a Varian Gemini 600 NMR spectrophotometer (Varian, USA) in methanol, chloroform.

Plant collection

During the month of July in 2013, leaves and branches of plant

materials were collected from Dir, Pakistan and identified and authenticated by Professor Whang Wan Kyunn. Plant specimen was submitted at the pharmaceutical resources botany laboratory in the college of Pharmacy, Chung-Ang University.

Method of extraction and isolation

Leaves and branches of plant was air dried and then powdered. Air dried powdered of plant (350 gm) was extracted three in 1 L of methanol under sonication. Filtration was done before the extract was evaporated under vacuum and amount yielded methanol extract was 4.75 gm. The 95%aqueous methanol extract was partitioned with n-hexane F1. Both layers were evaporated to dryness. Further, the dried methanol layer is dissolved in water and then partitioned with DCM (F2), EA (F3), and n-Butanol (F4) and water (F5) successively. Compounds were isolated on the basis of activity. The fractional F1 was chromatographed on silica gel by elution with hexane: EA which gave compounds 1 and 2. The fraction F3were chromatographed on sephadex columngave compound 3 and 4. Fraction F5 was chromatographed on sephadex and eluted with 20-40% MeOH to give six sub-fractions (Fr. 5-A-F) Fr. 5-E was chromatographed over sephadex to yield compound 5 (Rosmarinic acid). Water fraction F6 was chromatographed on sephadex and eluted with 20, 40, 60, 80 and 100% successively to obtained three sub-fractions (Fr. 6-A-C). The fraction Fr. 6-B was further chromatographed over sephadex column to yield compound 6 (4, 5 Dicaffeoyl Quinic acid). All isolated compounds were further purified by prep TLC. As a result 6 compounds were isolated

Identification and quantification of isolated compounds: Identification was done by NMR spectroscopy. Finger print analysis was also done by already prescribed method [16] with some modification. Quantitative analysis was carried out in both Pakistan thyme as well as Korean thyme. 1 gram of each powder was dissolved in 100 ml Methanol for examination of pattern. Pattern analysis was investigated with RP-C18 kromasil column (250 * 4.6, 5 µm) with gradient elution of two mobile phases. Solvent A was MeOH while solvent B was 0.05% formic acid in water were used in gradient A:B ≠ 30:70 → 40:60 (0 →10 min); 40:60 → 45:55 (10 → 30 min); 45:55 → 60:40 (30 → 32 min); 60:40 → 80:20 (32 → 40 min); 80:20 → 85:15 (40 → 60 min) at a wavelength of 280 nm with flow rate 0.8 ml/min and injection volume 20 µl. Analysis of peak was done by using standard.

Antioxidant Assay

DPPH assay

Due to presence of phenolic compounds, antioxidant activity was measured. The antioxidant activity of different fraction of TL and isolated compounds 3-6 were investigated by the method prescribed [17] with some modification. 20 µl of each sample (250, 500 and 1000 µg/ml for fraction, 12.5, 25, 50, 100 µM for the isolated compounds) were mixed with 180 µl of 0.1 mM DPPH solution with different concentration of samples. For negative control, Methanol was used. Ascorbic acid as well as trolox was used as positive control. After 30 mint of incubation at 37C, DPPH free radical was assessed by measuring the absorbance at 517 nm. The lower absorbance showed the higher free radical scavenging activity.

Observations were done in triplicate and percent inhibition of free radical scavenging DPPH was calculated by using following formula I% = Ac-At/Ac*100 where Ac represents the absorbance of the negative control reaction while At represents absorbance of the test sample and positive control. IC50 was calculated by plotting inhibition percentages versus sample concentrations.

Measurement of superoxide anion radical scavenging activity

Accumulation of uric acid results due to transferring of hypoxanthine to xanthine with the help of catalysis of enzyme xanthine oxidase. During oxidative stress, the xanthine oxidase activity has been reported to increase which in return produce uric acid and superoxide anion radicals. Because of Production of these superoxide anion radicals, reduction of NBT was done to mono as well as diformazan at an absorbance of 590 nm. Superoxide anion radical scavenging activity was measured according the method prescribed [18]. Altered concentrations (250, 500, 750 and 1000 µg/ml of fractions and 25, 50, 100, 200 µM of isolated compounds) samples were used. To the 20 µl of sample, added 160 µl of reaction solution which contained potassium phosphate buffer having (50 mM, pH 7.4), hypoxanthine (0.6 mM), NBT (0.2 mM), EDTA (1 mM). The reactions was started by adding 20 µl of XOD (200 mU/mL) and further incubated for 8min at 37˚C. Superoxide radical scavenging activity was calculated by following equation.

Superoxide anion radical scavenging activity (%) = Ac-At/Ac*100

Where Ac represents the absorbance with negative control while at represents the absorbance of test sample as well as positive control. Allopurinol was used as positive control.

Measurements of ABTS radical scavenging activity

ABTS radical scavenging activity was carried out according to method prescribed [19] with some modification. Stock aqueous solution of ABTS (7.4 Mm) and Potassium per sulfate (2.6 mM) were prepared and mixed in equal volumes. Then mixture was kept in dark for 16 hrs. Priorto assays, Stock solution were diluted with methanol until the absorbance at 732 nm was 0.8-1.2. 50 µl of Different concentrations of isolated compounds (25, 50, 100, 250 µM) and fractions (250, 500 and 1000 µg/ml) were used as test samples in 950 µl of $ABTS^{+}$ solution and absorbance was recorded at 732 nm using the spectrophotometer. AA and trolox were used as positive control. With the following equation ABTS radical scavenging activity was considered.

ABTS radical scavenging activity (%) = Ac-At/Ac*100.

Anticholinesterase Assays

Anticholinesterase assays was performed according to method prescribed by Ellman s assay with some modification. AChE was diluted to 0.3 U/ml in 20 mM tris HCL. While 0.5 mM of DTNB in tris HCL,0.6 mM of ATchI and O.1 mM of physostigmine solutions were prepared in distilled water. AChE (40 µL) was added to each sample (20 µL) and pre-incubated for duration of 30 min on ice. The reaction was initiated by adding the 20 µL DTNB and ATchI each and incubated at 37˚C for 20 min. Same steps were performed for positive physostigmine and negative control. After that, optical density was measured at 412 nm. Whole observations were performed in triplicate. Altered concentration of isolated compounds (5, 10, 20, 40 µg/mL) were used.

Statistical analysis

Whole data were represented as mean ± SD from three separate observations. The statistical analysis were performed by student's test and consider the difference are significant when $p<0.05$.

Results

Identification of isolated compounds

p-Cymene: NMR spectral data are in accordance with literature [20].

Oleanolic acid: H-NMR (600 MHz, CDCL3): δ 0.75, 0.77, 0.92, 0.93, 0.94, 0.98 (s, each 3H, CH3 × 6); δ 1.25 (s, 3H, H-27); δ 2.82 (dd, 1H, J = 3.66 and 13.6Hz, H-18); δ 3.21 (dd, 1H, J = 4.98 and 11.4 Hz, H-3), δ 5.24 (t, 1H, J = 3.78, H-12). C-NMR spectral data are in accordance with literature [21].

Luteolin (LN): Yellow crystalline powder. Rt = 43.17. HNMR (600MHz, CD3OD, C15H10O6): δ 6.54 (s, 1H, H-3); δ 6.21 (d, 1H, J = 2.1, H-6); δ 6.47 (d, 1H, J = 2.04, H-8) 8.56 (d, 1H, J = 2.5 Hz, H-2'), d 6.94 (s, 1H, H-3); δ 6.91 (d, 1H, J = 8.2, H-5'); δ 7.38 (dd, 2H, J = 2.1 and 8.2 Hz, H-6'); CNMR spectral data are according to literature data [22].

Salvianolic acid (SA): Light brown powder, Rt = 41.09, H-NMR (600MHz, CD3OD): Ring A; δ 6.91 (s, 1H, H-5); δ 7.28 (d, 1H, J = 11.1); Ring B δ 6.83 (d, 1H, J = 1.8, H-2); δ 6.67 (d, 1H, J = 7.86, H-5); δ 6.71 (dd, 1H, J = 1.8 and 7.8Hz, H-6); δ 3 .18 (dd, 1H, J = 3.18 and 14.46 Hz, H-7α); δ 3.05 (dd, 1H, J = 3.84 and 14.46 Hz, H-7ß); δ 5.20 (dd, 1H, J = 2.94 and 9.48 Hz, H-8); Ring C; δ 7.65(d, 1H, J = 1.68 Hz, H-2); δ 6.77 (dd, 1H, J = 2.1, and 8.04 Hz, H-5); δ 6.89 (d, 1H, J = 8.1 Hz, H-7) ; C-NMR spectral data are in accordance with to literature [2].

Rosmarinic acid (RA): Off-white powder, Rt = 31.33 mint, H-NMR (600MHz, CD3OD)δ 7.50 (d, 1H, J = 15.9 Hz, H-7); δ 7.03 (d, 1H, J=2.1 Hz, H-2'); δ 6.91 (dd, 1H, J=2.1 and 8.5 Hz, H-6); δ 6.76 (dd, 1H, J=8.5 Hz, H-5); δ 6.67, (d, 1H, J=8.04 Hz, H-5'); δ 6.62 (dd, 1H, j=2.01 and 8.1 Hz, H-6'); δ 6.26 (d, 1H, J = 15.9 Hz, H-8); δ 5.06 (dd, 1H, J = 3.3 and 9.8 Hz, H-8'); δ 3.08 (dd, 1H, J = 14.3 and 3.4 Hz, H-7'); δ 2.92 (dd, 1H, J = 9.8 and 14.4 Hz, H-7'); C-NMR spectral data are in accordance to literature [23].

Dicaffeoyl Quinic acid (DCQA): White powder, Rt = 19.12 mint, H-NMR (600MHz, CD3OD): δ 7.55 (d, 1H, J = 15.96, H-8); δ 7.05 (d, 1H, J = 1.98, H-2'); δ 6.95 (dd, 1H, J = 2.4 and 8.46 Hz, H-6); δ 6.79 (d, 1H, J = 8.1 Hz, H-5); δ 6.72 (d, 1H, J = 2.1 Hz, H-2); δ 6.70 (d, 1H, J = 7.98 Hz, H-5'); δ 6.57 (dd, 1H, J = 2.1 and 8.1 Hz, H-6'); δ 6.26 (d, 1H, J = 15.9 Hz, H-7); δ 5.20 (dd, 1H, J = 3.12 and 8.28 Hz, H-8'). C-NMR is in accordance with the literature [24].

Results

Result of finger print analysis and quantitative analysis of isolated compounds from Thymus linearis

Chromatograms of standard compounds (DCQA, IFA, RA, SA and LT), Pakistan thyme and Korean thyme are represented in Figure 1 eluted by HPLC. The chromatogram showed that standard compounds were also present in Korean thyme but they are quantitatively different from each other. Peaks identification was done by matching with the retention time (t_R) with standard compounds. The contents of standard substances in Korean and Pakistan thyme are listed in Table 1 which was used for antioxidant and anti-cholinesterase activity for further observations. Quantitative analysis showed that Korean thyme has more quantity of isolated phenolic compounds as compared to Pakistan.

Result of antioxidant assays

DPPH assay has been widely used in order to measure the antioxidant activity. DPPH radical reduction ability was investigated by the falling in its absorbance at a wavelength of 517 nm. The isolated compounds from *Thymus linearis* showed a concentration dependent antiradical activity by decreasing the stable radical DPPH to turn into yellow colored diphenylhypicrylhydrazine derivatives (Table 2 and Figure 2). All samples have antioxidant activity against DPPH and reducing power increased in dose dependent manner. The data exhibited the IC50 value for fraction at the dose of 1000 µg/ml for Hx, DCM, EA, n-BuOH and H_2O were measured. Among them EA, n-BuOH and H_2O showed the maximum antioxidant activity. The DPPH activity of isolated compounds was compared with control AA and trolox. The IC50 values of AA and Trolox at 100 µM were 36.22 ± 0.00 and 64.32 ± 0.00 respectively. Among them, DCQA showed the greatest activity against DPPH with an IC50 value of 34.93 ± 0.23 µM, followed by increasing in order SA, RA and LT (45.53 ± 1.42, 59.22 ± 0.28 and 126 ± 1.11 respectively). Data showed that DCQA has more significant value than with standard AA but less than to trolox. The DPPH radical scavenging activity of these four compounds are profound significant in comparison with ascorbic acid (AA) and trolox, positive control.

Hypoxanthine- xanthine oxidase system acts as a source of superoxide radical. IC50 values of isolated compounds were measured in this assay. The IC50 values ranged from 50.67 to 121.90 µM. The value of positive control, allopurinol, was 50.72 ± 0.42 µM. Isolated compounds exhibited significant superoxide radical scavenging inhibiting activity in dose dependent manner. DCQA exhibited greatest activity as, IC50, 68.57 ± 0.04 µM, followed by increasing in order SA, RA, LT. In comparison with the positive control, allopurinol, all the four compounds showed profound activity (Table 2 and Figure 3).

According to condition used in ABTS assay, the IC50 values of Ascorbic acid and Trolox were, positive control, 79.32 ± 1.69 and 87.81 ± 0.15 µM respectively. The IC50 values of isolated compounds were ranged from 51.66 µM to 123.18 µM. All the tested compounds have significant ABTS radical scavenging activity. Among them, DCQA showed the profound inhibition activity against ABTS as 51.66 ± 0.00 followed, increasing order, by SA, RA and LT (Table 2 and Figure 4). In ABTS assay, DCQA showed less IC50 value in comparison with positive controls.

Result of ACHE inhibition assays

In order to improve the cognitive function in patients suffered from AD by inhibiting the central cholinergic activity, isolated compounds were evaluated to check the acetyl cholinesterase inhibiting activity. Data obtained from IC50 observations showed that DCQA showed the strongest ACHE inhibition activity (37.47 ± 3.2) followed in increasing in order SA, RA and LT and percentage inhibition are presented (Table 2 and Figures 5-7). The anti-cholinesterase activity was observed in dose dependent manner. The data obtained from IC50 values of, positive control, phytostigmine was 1.68 ± 0.43.

Discussion

Numerous long-lasting and deteriorating diseases including cardiovascular disease, diabetes, cancer, ageing and neurodegenerative disease are due to the oxidative damage induce by free radical. Reactive oxygen species show a major character in this oxidative harm which leads to DNA mutation, protein inactivation, lipid peroxidation,

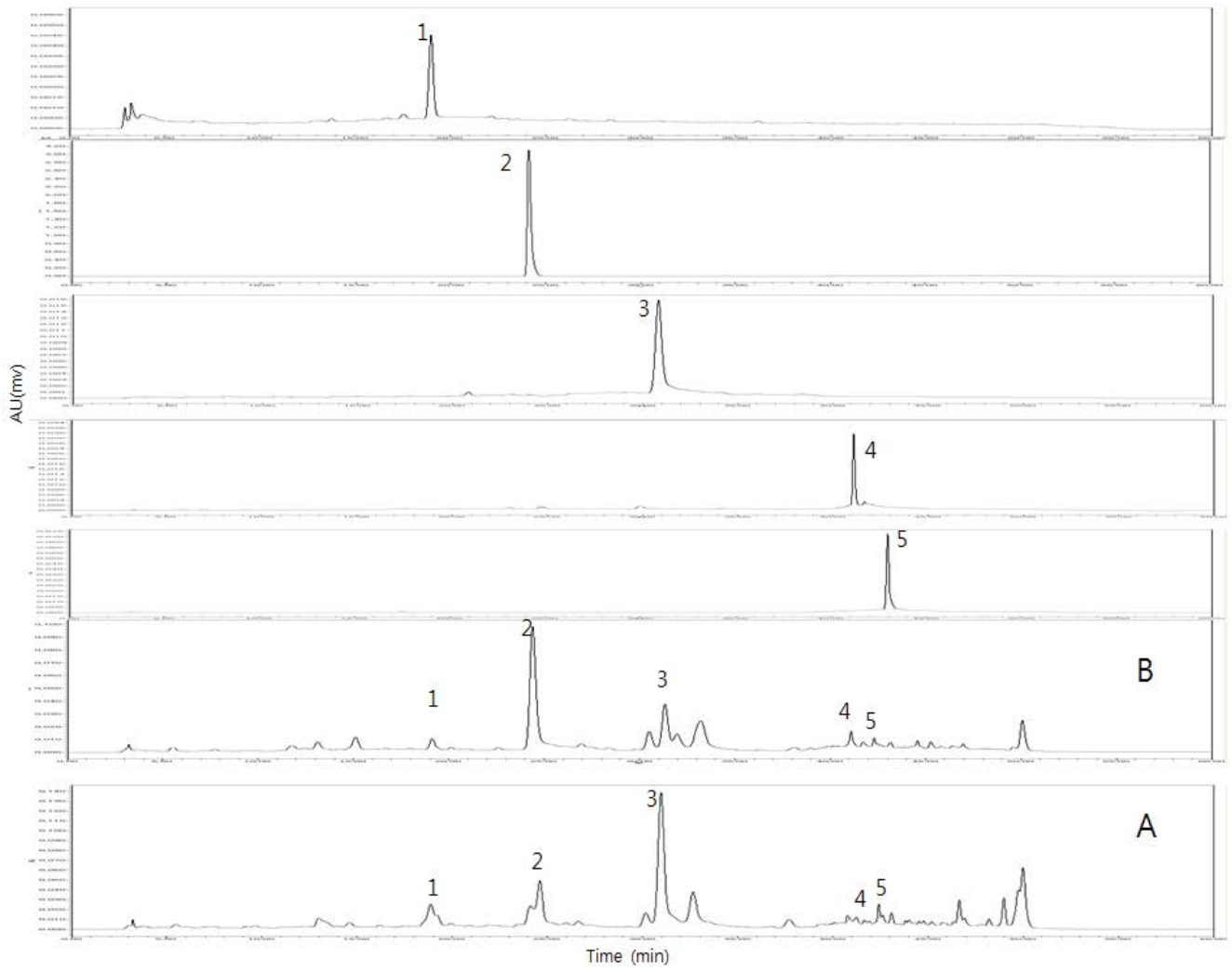

Figure 1: Chromatogram of the standard compounds, methanolic extract of Thymus from Korea (A) and Pakistan (B).

Table 1: Retention time of the examined compounds and their contents in leaves of Pakistan and Korean Thyme.

Peaks	Compounds	Retention time (min)	Contents in dry leaves (mg/g)	
			Pakistan	Korea
1	DCQA	19.12	2.73	8.77
2	IFA	24.39	3.22	1.42
3	RA	31.33	2.52	10.33
4	SA	41.09	1.04	0.69
5	LN	43.17	0.15	0.29

cell apoptosis and abnormal proliferation. Natural antioxidants are responsible for the preventing or inhibiting the toxic consequence of oxidative stress. Free radical scavengers such as polyphenol, flavonoids and phenolic compounds are present in herbs, fruits, vegetables and spices [25]. The potentiation of phenolic compounds isolated from TL to inhibit the ACHE showed the importance of thyme as pharmacotherapy for treatment of AD. The herb thyme is very well known to have capacity of antioxidant activity owing to the existence of phenolic compounds such caffeic acid, cinnamic acid, rosmarinic acid, ferulic acid, chlorogenic acid etc. [26]. However, some studies suggest that the presences of polyphenols are considered very important features of herbal drugs. Quantitative analysis showed the presence of compounds in both the extract but exhibited different amount. Among the isolated compounds, highest quantity of RA and DCQA was found in extract of thymus of Korean origin. While in Pakistan thyme, IFA was in highest quantity. Due to presence of high quantity of RA and DCQA in Korean thyme can show more antioxidant and ACHE inhibiting activity as compared to Pakistan thyme thyme. Therefore, presence of high amount of phenolic compounds can play a vital role play role in many pharmacological effects such as antioxidant, diuretic activity and anti-inflammatory [27]. In comparison with standards, among the isolated compounds

Table 2: IC50 values of compounds on DPPH, Superoxide anion assay, ABTA assay and ACHE assay. All observation data are expressed in mean ± SD in triplication. *p 0<.05, **p<0.01 significant versus control. nt (not detected).

Treatment/ dose	DPPH assay IC50 (µM)	Superoxide anion scavenging IC50 (µM)	ABTS assay IC50 (µM)	ACHE inhibitory activity (µM)
Luteolin (LN)	126 ± 1.11	121.95 ± 0.04	123.18 ± 4.6	80.37 ± 1.57
Salvianolic acid (SA)	45.53 ± 1.42	98.26 ± 0.7	73.61 ± 1.63	45.93 ± 1.84
Rosmarinic acid (RA)	59.22 ± 0.28	108.45 ± 0.06	74.39 ± 4.72	58.09 ± 0.29
4,5-Dicaffeoyl-quinic acid (DCQA)	34.93 ± 0.23	68.57 ± 0.04	51.66 ± 0.10	37.47 + 3.2
Ascorbic acid	36.22 ± 1.19	nt	79.32 ± 1.69	nt
Trolox	64.32 ± 0.45	nt	87.81 ± 0.15	nt
Allopurinol	nt	50.72 ± 0.42	nt	nt
Physostigmine	nt	nt	nt	1.68 ± 0.43

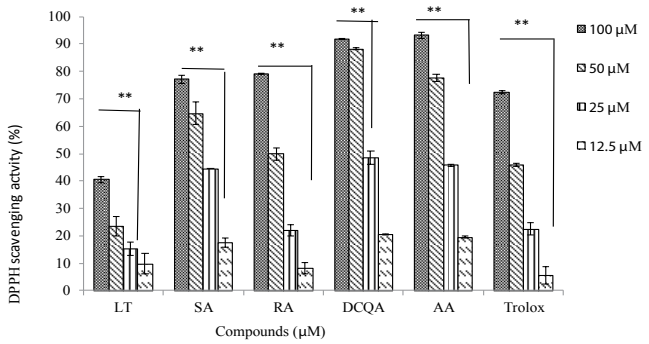

Figure 2: DPPH Radical Scavenging activity (%) of different fraction of isolated compounds and positive control groups from *Thymus linearis* in comparison with dose (µM). All observation data are expressed in mean ± SD in triplication. *p<0.05, **p<0.01 significant versus control.

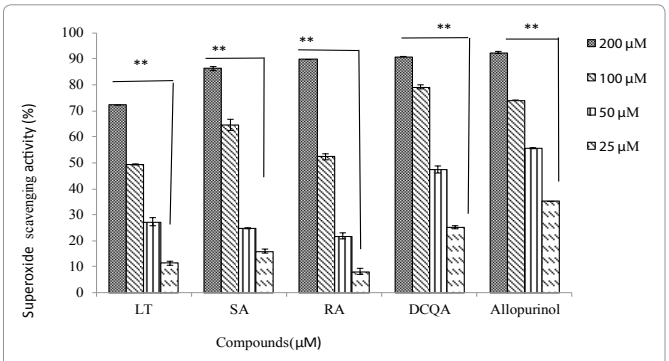

Figure 3: Superoxide Radical Scavenging activity (%) of isolated compounds from *Thymus linearis* in comparison with dose (µM). All observation data are expressed in mean ± SD in triplication. *p<0.05, **p<0.01 significant versus control.

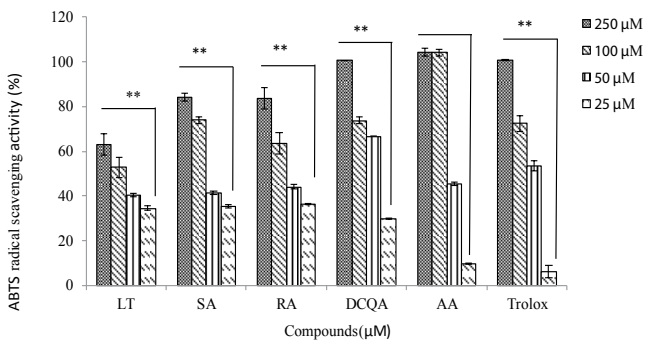

Figure 4: ABTS Free Radical Scavenging activity (%) of isolated compounds from *Thymus linearis* and positive controls in comparison with dose (µM). All observation data are expressed in mean ± SD in triplication. *p<0.05, **p<0.01 significant versus control.

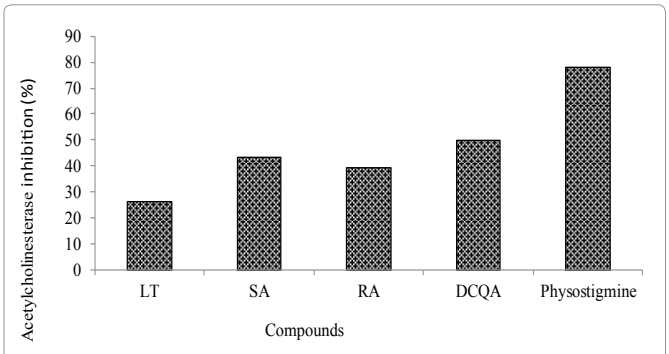

Figure 5: Acetylcholinesterase inhibition (%) of major components (40 µg/mL) from *Thymus linearis* and Physostigmine (10 µM). All observation data are expressed in mean ± SD in triplication. *p<0.05, **p<0.01 significant versus control.

SA, RA and DCQA showed profound significantly results from antioxidant activity as well as anti-cholinergic assays which exhibited the importance of theses phenolic compounds in combating the disease due to oxidative stress and neurodegenerative diseases. Therefore it can be suggested that thymus linearis containing phenolic compounds can be effective against oxidative stress related diseases and AD.

Conclusion

In conclusion, it can be described that *Thymus linearis* contains phenolic compounds which are related with strong antioxidant and anticholinergic activities. So, it can provide protection for biological system against the diseases such as cancer, diabetes, rheumatoid arthritis due to oxidative stress at cellular level. Therefore, it is stated that by using the *Thymus linearis* in food as a dietary natural antioxidant supplement, many harmful diseases can be protected due to the high content of phenolic component which have potential antioxidant and ACHE inhibiting activities.

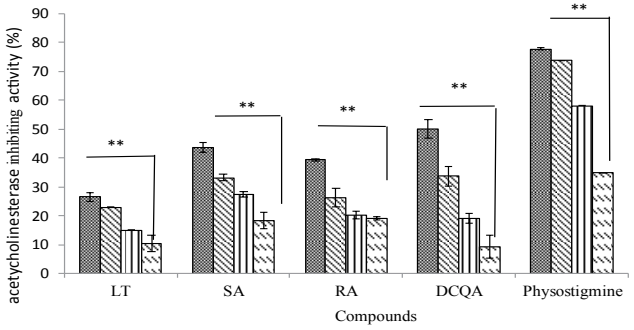

Figure 6: Dose dependent manner of Acetyl cholinesterase inhibition (%) of major components in comparison with control. All observation data are expressed in mean ± SD in triplication. *p<0.05, **p<0.01 significant versus control.

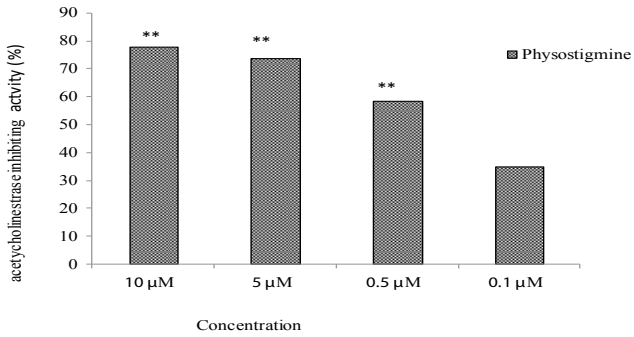

Figure 7: Acetyl cholinesterase inhibition (%) of Physostigmine in comparison with dose (µM).

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