

In vitro Antioxidant Activity of Two Selected Herbal Medicines

S. Vinotha, I. Thabrew, S. Sri Ranjani

Abstract—Hot aqueous and methanol extracts of the two selected herbal medicines such as Vellarugu chooranam (V.C) and Amukkirai Chooranam (A.C) were examined for total phenolic and flavonoid contents and *in vitro* antioxidant activity using four different methods. The total phenolic and flavonoid contents in methanol extract of V.C were found to be higher (44.41±1.26mg GAE/g; 174.44±9.32mg QE/g) than in the methanol extract of A.C (20.56±0.67mg GAE/g; 7.21±0.85mg QE/g). Hot methanol and aqueous extracts of both medicines showed low antioxidant activity in DPPH, ABTS, and FRAP methods and Iron chelating activity not found at highest possible concentration. V.C contains higher concentrations of total phenolic and flavonoid contents than A.C and can also exert greater antioxidant activity than A.C, although the activities demonstrated were lower than the positive control Trolox. The *in vitro* antioxidant activity was not related with the total phenolic and flavonoid contents of the methanol and aqueous extracts of both herbal medicines (A.C and V.C).

Keywords—Activity, Different extracts, Herbal medicine, *in vitro* antioxidant.

I. INTRODUCTION

FOR thousands of years, mankind has known about the benefit of drugs from nature. Plant extracts, for the treatment of various ailments, were highly regarded by the ancient civilizations. Even today, plant materials remain an important resource for combating illnesses [1]. Antioxidants mop up damaging chemicals in the body and guard against many chronic diseases. Heart disease, arthritis, cancer and many other chronic diseases derive from the same source: fortuitous mutations caused largely by free radicals. The cells are protected from decomposition by ensuring sufficient intake of antioxidants daily [2].

The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability, in recent years one of the areas, which attracted a great treaty of attention, is antioxidant in the control of degenerative disease in which oxidative dent has been implicated. Several plant extracts have been shown to antioxidant activity [3].

Herbal drugs are rapidly becoming popular in recent year as

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an alternative therapy. Numerous single formulations, which are single herbal extracts/ fractions, are used for the treatment of rheumatic diseases. Antioxidants that can protect joints from oxidative damages are included in single formulations. For developing a satisfactory antioxidant herbal formulation, there is a need to evaluate the formulation for desired properties such as antioxidant activity. The desired activities of the single herbal formulations containing plants/extracts have to be tested again in the formulation form [4].

Vellarugu Chooranam (V.C) and Amukkirai Chooranam (A.C) (Fine powder preparation for internal use) are important single herbal preparations which are mentioned in the Traditional medical literatures in Sri Lanka for the treatment of inflammatory conditions and variety of musculoskeletal conditions such as rheumatism, back pain, arthritis, and etc. The main components of the V.C and A.C are whole plant powder of the *Enicostemma littorale* Blume and root powder of *Withania somnifera* Dunal [5], [6].

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of different extracts of plants and plant products. In this present study, hot aqueous and methanol extracts of these two herbal medicines were examined for total phenolic and flavonoid contents and *in vitro* antioxidant activity using four different methods such as DPPH radical scavenging, FRAP, ABTS and Iron chelating activity.

II. MATERIALS AND METHODS

A. Plant Material

Whole plants of *Enicostemma littorale* were collected during the month of October 2011 to January 2012 in and around Jaffna District. Roots of *Withania somnifera* were purchased from a reputed vendor of herbal material in Jaffna District, Sri Lanka.

These plants (*Withania somnifera* & *Enicostemma littorale*) were botanically authenticated and voucher specimens (Assess. No. 2453 & 2454) were deposited in the Bandaranayaka Ayurveda Memorial Research Institute, Nawinna, Maharahama, Sri Lanka.

B. Preparation of Herbal Medicine

The collected *E. littorale* whole plants were cut in to small pieces and washed with tap water. The purchased *W. somnifera* roots were cut in to small pieces and boiled with cow's milk (1:1w/v). These plant materials were air-dried thoroughly under shade (at room temperature) for 2-3 weeks to avoid direct loss of phyto-constituents from sunlight. The shade dried materials were powdered using the pulverizer and

sieved up to 80 meshes separately. It was then homogenized to fine powder and stored in air-tight container for further analysis.

C. Chemicals

All chemicals used were of at least analytical grade. Folin-Ciocalteu reagent, gallic acid, quercetin krist ($C_{15}H_{10}O_7 \cdot 2H_2O$), 6-hydroxy-2-5-7-8-tetramethylchroman-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulphate, 2,4,6-tripyridyl-s-triazine (TPTZ), 4,4'-disulfonic acid sodium salt (ferrozine) and Ethylenediamine tetra acetic acid disodium salt dehydrate (EDTA) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Other reagents were obtained from Fisher Scientific (Springfield, NJ, USA). All the analyses were carried out using High-throughput 96-well micro-plate reader (Spectra Max Plus384, Molecular Devices/ U.S.A).

This study was carried out with assistance of authorizing officer at Bioactivity Lab/ Herbal Technology Section, Industrial Technology Institute, Colombo- 07, Sri Lanka.

D. Preparation of Freeze-Dried Extracts

Ten gram of each herbal preparation was suspended in 150ml distilled water and refluxed three hours in a round bottom flask on heating mantle separately. The raw extracts were pooled, filtered, and evaporated to dryness in a rotary vacuum evaporator at 40°C. The dried crude aqueous extracts were weighed and 1 g portions of each were freeze dried and stored at 4°C for further investigation. Same procedure was followed using 95% (v/v) methanol to dried crude methanol extract. The extracts were dissolved in DMSO (Dimethyl sulfoxide) prior to use.

The extractable matters were calculated as the content of in mg per g of air-dried material according to the way of wet and dry basis.

E. Assaying Methods

1. Total Phenolics Contents Determination

The total phenolics content in hot aqueous and methanol extracts of each herbal preparation were estimated according to the Folin–Ciocalteu method [7]. The freeze dried aqueous extracts were re-dissolved with DMSO and distilled water using Vortex machine (VIBROFIX VF1 Electronic) and the concentrations of each extract adjusted to 1mg/ml with distilled water. Each methanol extract was re-dissolved with DMSO and methanol (Anala R* grade) to a concentration of 1mg/ ml. After 10 minutes, 40 µL of each extracts, and 110 µL of 10-fold diluted 2N Folin–Ciocalteu reagent (Sigma - UK) were combined in a micro plate as triplicate and then mixed well using a Vortex mixture (Biocote - stuart). The mixture was allowed to react for 5 minute then 70µl of 10% Sodium carbonate (Na_2CO_3) solution was added and mixed well. The solution was incubated at room temperature (27°C) in the dark for 30 minutes. The absorbance was measured at 765nm against a methanol blank, using a Micro Plate Reader

(Molecular Deviser – Spectra Max 384 Plus). Gallic acid (Sigma- Aldrich Chemical, USA) (0.125- 1mg/ml) was used as a standard to prepare a calibration curve. The total phenolic content was expressed in mg of Gallic acid equivalents (GAE/g) of extract and the values are presented as means of triplicate analyses.

2. Total Flavonoids Contents Determination

The total flavonoids content in hot aqueous and methanol extracts of each herbal preparation were estimated according to the $AlCl_3$ method [8]. Each dried aqueous and methanol extracts were re-dissolved in methanol to a final concentration of 5mg/ml. 100µl of 2% Aluminium chloride ($AlCl_3 \cdot 6H_2O$) in methanol mixed with 100µl of 2.5 times and 5 times diluted each extracts in methanol (2.5mg/ml and 5mg/ml) were combined in a micro plate as triplicate and then mixed well using a Vortex mixture. The mixture was allowed to react for 10 minutes. The absorbance was measured at 415 nm against a methanol blank using a Micro Plate Reader. The results were determined using a standard curve prepared with Quercetin (Sigma- Aldrich Chemical, USA) 1mg/ ml, 6-fold diluted with MeOH} as the standard. The total flavonoid contents are expressed as mg of quercetin equivalent (QE)/g of extract.

3. Iron Chelating Activity

The chelation of iron (II) ions by the different extracts was carried out as described by standard methods [8], [9]. One hundred micro-liters (100µl) of each extract (5 concentrations) were added to 40 µl water in the micro plate as triplicate and the pre plate reading at 562nm was recorded using a Micro Plate. Then 20 µl of 1mM $FeSO_4 \cdot FeSO_4$ was added in to the different concentrations of the extracts. The controls contained all the reaction reagents except the extract or positive control substance. After 5 min incubation, the reaction was initiated by the addition of 40µl of 1mM ferrozine solution. After a 10 min. equilibrium period, the absorbance at 562nm was recorded. The increased sensitivity obtained from the stable magenta colour of the iron-ferrozine complex makes it possible to monitor iron chelating activity of extracts. The iron chelation activities were calculated from the absorbance of the control (Ac) and of the sample (As) using Equation and expressed as EDTA equivalents (mg EDTA/g extract). EDTA was used as positive control. The values are presented as the means of triplicate analyses.

$$\text{Inhibition \%} = \frac{Ac - As}{Ac} \times 100$$

4. DPPH Radical Scavenging Assay

The ability of the extracts to scavenge DPPH free radicals was determined by the standard method [10]. A 100µl various concentrations of each extract was mixed with 100µl methanol buffer and 50 µl of 0.05mM 1, 1-diphenyl-2-picrylhydrazyl in methanol. The controls contained all the reaction reagents except the extract or positive control substance. After 20 min incubation in darkness and at 25°C, the resultant absorbance was recorded at 517nm. Linear graph of concentration Vs percentage inhibition was prepared and IC50 values were

calculated. The % inhibition was calculated according to the following equation.

$$\text{DPPH (Inhibition \%)} = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100$$

IC50 was calculated from equation of line obtained by plotting a graph of concentration versus % inhibition. The values are presented as the mean of triplicate analyses. Trolox was used as positive control. The antioxidant capacity based on the DPPH free radical scavenging ability of the extracts were expressed as μmol Trolox equivalents per gram of plant material on dry basis.

5. Ferric Reducing Antioxidant Potential Assay

The ability to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain [11]. A 20 μl various concentrations of each extract was mixed with 30 μl of 300mM, pH 3.6 acetate buffer and 150 μl of FRAP reagent (10 parts of 300mM sodium acetate buffer at pH 3.6, 1 part of 10mM TPTZ solution in 40mM HCl and 1 part of 20mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution) and the reaction mixture was incubated in a water bath (WISE BATH[®], WISD Laboratory Instrument) at 37°C for 10 min. The increase in absorbance at 600 nm was measured at 30 min. against a blank that was prepared using acetate buffer. Trolox was used as positive control. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as μmol Trolox equivalents per gram of plant material on dry basis.

6. ABTS Radical Scavenging Assay

ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, assay is based on the scavenging of light by ABTS radicals. ABTS radical cation (ABTS⁺) was produced by reacting ABTS solution (7mM) with 2.45mM potassium persulfate and the mixture was allowed to stand in dark at 25 \pm 2°C for 12-16 h before use. For this study, different concentrations (100 $\mu\text{g}/\text{ml}$) of extracts (150 μl) were mixed with 120 μl of 5mM, pH 7.4 phosphate buffer and 200 μl of diluted ABTS solution and the reaction mixture was incubated at 25°C for 10 min. The absorbance was read at 734nm and the experiment was performed in triplicate. Linear graph of concentration Vs percentage inhibition was prepared and IC50 values were calculated. The antioxidant capacity based on the ABTS free radical scavenging ability of the extracts were expressed as μmol Trolox equivalents per gram of plant material on dry basis [12].

F. Statistical Analysis

All analyses were performed in triplicate, and the data are expressed as the mean \pm standard deviation (SD). Data were analysed by statistical software-Statistical Package for Social Sciences (SPSS) version 17.

III. RESULTS AND DISCUSSIONS

A. Total Phenolics and Flavonoids Contents

Table I shows an overview of extraction yields, total phenolics and flavonoids contents for the hot aqueous and methanol extracts of the V.C and A.C.

TABLE I
EXTRACTION YIELD, TOTAL PHENOL AND FLAVONOID CONTENT OF THE
VELLARUGU AND AMUKKIRAI CHOORANAM

Name of the herbal material and its extract	Extraction yield (mg/ g)	Total polyphenolic content (mg GAE/ g \pm SD)	Total polyphenolic content (mg GAE/ g \pm SD)
<i>Vellarugu chooranam</i>			
1. Hot aqueous extract	362.9	20.11 \pm 0.92	42.74 \pm 2.82
2. Hot methanol extract	217.6	44.41 \pm 1.26	174.40 \pm 9.32
<i>Amukkirai chooranam</i>			
1. Hot aqueous extract	291.2	9.90 \pm 0.42	2.74 \pm 0.35
2. Hot methanol extract	135.0	20.56 \pm 0.67	7.21 \pm 0.85

Values are expressed as mean \pm S.D., n = 3.

Polyphenolics content are expressed as Gallic acid equivalents (GAE) while total flavonoid contents are expressed as Quercetin equivalents (QE).

The percentage yields of hot aqueous and methanol extractive values (36.2 & 21.7%) of V.C was greater than the percentage yield of aqueous and methanol extractive values (29.1 & 13.5%) of A.C. No significant association could be found neither between the extraction yields with total phenols and flavonoids nor between the extraction yields with the results from the different antioxidant assays.

In most of the antioxidant study the phenolic compounds was directly correlated with its antioxidant ability. Phenols and poly phenolic compounds, such as flavonoids are widely found in food products derived from plant sources and they have been shown to possess significant antioxidant activities [13], [14]. Therefore, the amount of total phenols in the extracts was investigated by the Folin-Ciocalteu method. The content of total phenols is expressed as gallic acid equivalents (mg GAE/g dry extract). The total phenolics content in decreasing order was Methanol extract of V.C > Methanol extract of A.C > Aqueous extract of V.C > Aqueous extract of A.C are shown in Fig. 1. Aqueous extract of A.C had the lowest content of total phenolic content.

This may be due to polarity of the solvent. The extraction yields, nature of the compounds, and the materials from which the compounds were extracted strongly depend on the solvents due to the presence of different concentration of bioactive compounds with different polarities [15].

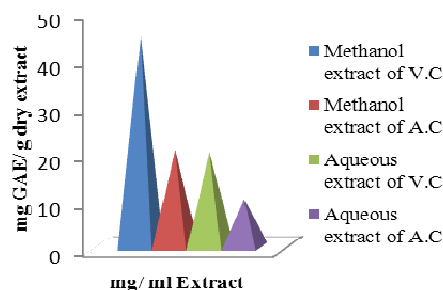


Fig. 1 Total phenolics contents of different extracts of Vellarugu (V.C) and Amukkirai chooranam (A.C).

Kessler et al., 2003 have been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable [16]. Ebrahimzadeh et al., 2008 stated that the compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for antioxidant effect in the plants [1]. The content of total flavonoids is expressed as quercetin equivalents (mg QE/g dry extract). Fig. 2 represents, the total flavonoid content in decreasing order was Methanol extract of V.C>Aqueous extract of V.C>Methanol extract of A.C>Aqueous extract of A.C. Aqueous and methanol extracts of A.C had the lowest content of total phenolic content.

Total phenolics and flavonoids contents of V.C were higher than A.C.

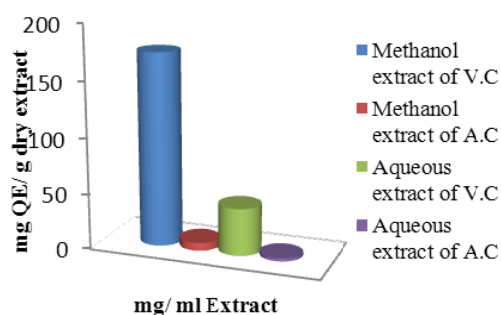


Fig. 2 Total flavonoids contents of different extracts of Vellarugu (V.C) and Amukkirai chooranam (A.C).

B. Iron Chelating Activity

The chelating of Fe^{2+} by extracts was estimated by the method of Carter, 1971 [9]. Ferrozine can quantitatively form complexes with Fe^{2+} . However, in the presence of chelating agents, the complex formation is disrupted with the result that the magenta colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals [1]. In this present activity the magenta colour of the complex was not decreased and values were lower than limit. Hot aqueous and methanol extracts of V.C and A.C, interfered with the formation of ferrous and ferrozine complex, suggesting that these all extracts have not ferrous chelating activity at 1.75 mg/ml concentration (highest possible concentration).

C. DPPH Radical Scavenging Activity

The stable radical DPPH has been used widely for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials [17]. The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 517 nm. In this study, the antioxidant activity was expressed as Trolox equivalents per gram of plant material on a dry basis as it is a more meaningful and descriptive expression than assays that express

antioxidant activity as the percentage decrease in absorbance. The DPPH is a stable nitrogen-centered free radical the colour of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [13].

Fig. 3 represents the methanol and aqueous extracts of V.C and A.C displayed DPPH radical scavenging activities but varying order. Maximum activity was shown by methanol extract of V.C and minimum by aqueous extract of A.C. The radical scavenging potential of the preparations used here were does dependent that on increasing the concentration of extracts (156 – 625 μ g/ml). The highest activity shown by methanol extract of V.C was 71.98% at 625 μ g/ml whereas highest antioxidant activity shown by methanol extract of A.C, aqueous extracts of V.C and A.C were 44.06%, 29.43% and 5.57% respectively, very low as compared to methanol extract of V.C.

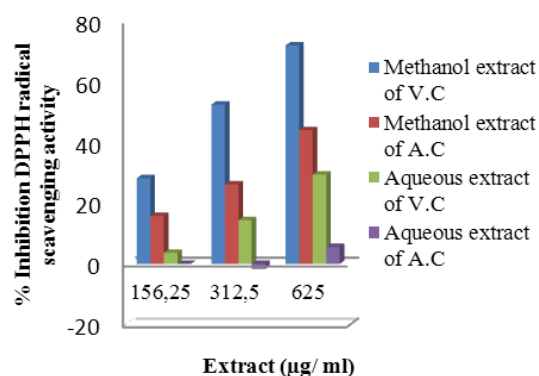


Fig. 3 IC 50 values and DPPH radical scavenging activity of the Vellarugu and Amukkirai Chooranam

Table II shows an overview of IC 50 values, DPPH radical scavenging activity for the hot aqueous and methanol extracts of the V.C and A.C.

TABLE II
IC 50 VALUES AND DPPH RADICAL SCAVENGING ACTIVITY OF THE VELLARUGU AND AMUKKIRAI CHOORANAM

Name of the herbal material and its extract	IC 50 values μ g/ml	DPPH antioxidant activity μ mole TE/ml
<i>Vellarugu chooranam</i>		
Hot aqueous extract	1207.00	23.84 \pm 01.24
Hot methanol extract	297.83	134.11 \pm 20.70
<i>Amukkirai chooranam</i>		
Hot aqueous extract	5442.00	5.89 \pm 0.63
Hot methanol extract	791.00	41.31 \pm 4.28
Trolox as standard	7.38	

Values expressed as mean \pm S.D., n=5 different concentrations with three replicates.

Antioxidant activity expressed as Trolox equivalents per gram of plant material on a dry basis.

Although the methanol and aqueous extracts of both preparations showed weak antioxidant activity in comparison

to the standard Trolox, the antioxidant activity of the V.C higher than that of A.C.

C. FRAP Assay

At low pH, measuring the change in absorption at 600 nm can monitor reduction of a ferric complex to the ferrous form, which has an intense bluish green color. The change in absorbance is directly related to the combined or "total" reducing power of the electron-donating antioxidants present in the reaction mixture. Here the FRAP showed the results of aqueous and methanol extracts that of μ mole equivalent to Trolox (TE)/ g of sample. The FRAP assay provides a direct estimation of the level of antioxidants present in a sample and is based on the ability of analyze to reduce the Fe^{3+}/Fe^{2+} pair. An antioxidant capable of donating a single electron to the ferric-TPTZ (Fe (III)-TPTZ) complex would cause the reduction of this complex into the blue ferrous-TPTZ (Fe (II)-TPTZ) complex which absorbs strongly at 600 nm. The FRAP values ranged from 244 -1266 μ mole TE/ g of dry mater are shown in Table III.

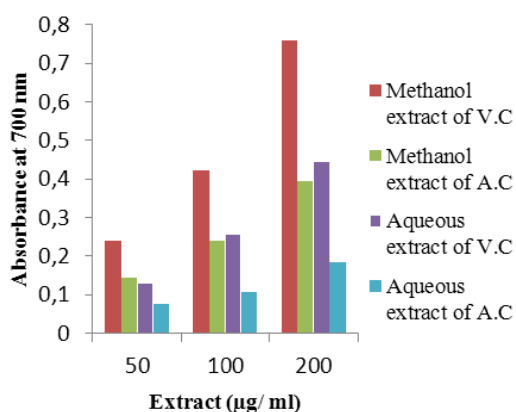
TABLE III

FRAP ACTIVITY OF THE VELLARUGU AND AMUKKIRAI CHOORANAM	
Name of the herbal material and its extract	FRAP antioxidant activity μ mole TE/ml
<i>Vellarugu chooranam</i>	
Hot aqueous extract	677 \pm 24
Hot methanol extract	1266 \pm 22
<i>Amukkirai chooranam</i>	
Hot aqueous extract	244 \pm 07
Hot methanol extract	659 \pm 19

Values expressed as mean \pm S.D., n=5 different concentrations with three replicates.

Antioxidant activity expressed as Trolox equivalents per gram of plant material on a dry basis.

When considering in this assay the methanol and aqueous extracts, the following decreasing order was found: Methanol extract of V.C > Aqueous extract of V.C > Methanol extract of A.C > Aqueous extract of A.C (Fig. 4). In this assay, the FRAP activity of the V.C higher than that of A.C.

Fig. 4 FRAP activity of the *Vellarugu* and *Amukkirai Chooranam*

D. ABTS Activity

The original ABTS^{•+} assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of

ABTS to produce the radical cation, in the presence or absence of antioxidants. This has been criticized on the basis that faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical. A more appropriate format for the assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction ns with putative antioxidants [12]. The ABTS assay measured ability of direct production of the blue/ green ABTS^{•+} chromospheres through the reaction between ABTS and potassium per sulfate.

Table IV summarizes the ABTS activity of the methanol and aqueous extracts of both preparations.

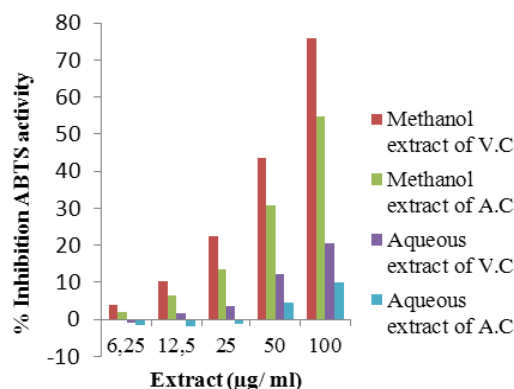
TABLE IV
IC 50 VALUES AND ABTS RADICAL CATION DECOLORIZATION ASSAY OF THE VELLARUGU AND AMUKKIRAI CHOORANAM

Name of the herbal material and its extract	IC50 values μ g/ml	ABTS antioxidant activity μ mole TE/ g
<i>Vellarugu chooranam</i>		
Hot aqueous extract	224.0	88.42 \pm 18.52
Hot methanol extract	63.41	389.07 \pm 25.56
<i>Amukkirai chooranam</i>		
Hot aqueous extract	401.0	38.95 \pm 19.9
Hot methanol extract	89.42	253.80 \pm 04.28
Trolox as standard	06.75	

Values expressed as mean \pm S.D., n=5 different concentrations with three replicates.

Antioxidant activity expressed as Trolox equivalents per gram of plant material on a dry basis.

The methanol and aqueous extracts of both preparations showed lower antioxidant activities in comparison to the standard Trolox. Methanol extract of V.C showed higher activity (IC 50: 63.41 μ g/ ml) and aqueous extract of A.C showed the weakest (IC 50: 401 μ g/ ml). The IC 50 value for Trolox was 6.5 mg/ ml. The following decrease order was obtained with methanol and aqueous extracts of both preparations: Methanol extract of V.C > Methanol extract of A.C > Aqueous extract of V.C > Aqueous extract of A.C (Fig. 5).

Fig. 5 IC 50 values and ABTS radical scavenging activity of the *Vellarugu* and *Amukkirai Chooranam*

V.C contains higher concentrations of total phenolic and flavonoid contents than A.C and can also exert greater antioxidant activity than A.C, although the *in vitro* antioxidant

(ABTS, FRAP and DPPH) assays demonstrated were lower than the positive control Trolox. The activity was not related with the total phenolic and flavonoid content of the methanol and aqueous extracts of both medicines.

IV. CONCLUSION

The results of present study revealed that hot methanol and aqueous extracts of V.C possessed high antioxidant activity than A.C. These preparations further investigations of other standard *in vitro* antioxidant assays and isolate the active constituents, especially due to their non-polar nature should be carried out in future.

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