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# Analysis of In Vitro Antioxdant Potential of Five Diiferent Solvent Fractions of Methanolic Leaf Extract Along with Identification of Specific Phytochemicals of *Trichosanthes dioica* And *Typhonium trilobatum* (Less Focussed Edible Medicinal Herb)

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**Abstract :** Polyphenols and flavonoids, a subclass of phytochemicals are able to scavenge free radicals by H- atom transfer and thus able to reduce the noxious effects due to oxidative stress. Phytochemicals are secondary metabolites of plant which possesses medicinal value and they can modulate a wide range of physiological function. So, modern research has been focussed to indentify bioactive compounds from plant source. *Trichosanthes dioica* and *typhonium trilobatum* are two edible plant which have promising place in traditional medicine but lack of scientific evidence remain them less focussed. The objective of present study is to identify the specific phytochemicals of them and radical scavenging potential of that phytochemicals. The crude methanolic leaf extracts of samples are fractionate by five different solvent of increasing polarity and in vitro antioxidant activity in term of DPPH, ABTS radical scavenging potential, Ferric reducing ability potential (FRAP), total antioxidant capacity (TAC) of each fraction were measured. Each fraction subjected to thnlayer chromatography to identify the specific phytochemicals. Result suggests that both the samples are potential source of antioxidant due to the presence of specific phytochemicals such as gallic acid and quercetin confirmed by TLC. Among different solvent fraction most active is diethyl ether followed by aqueous and ethyl acetate. It suggest that most active phytochemicals of these plants are moderately followed by highly polar. These findings may help the future studies to isolate natural antioxidant and formulate drug from the samples.
**Keywords** : Antioxidant, Free radical, Phytochemicals, DPPH, Thin layer chromatography.

## Introduction

Free radicals can induce the onset of various diseases, e.g.,cancer, rheumatoid, arthritis and arteriosclerosis as well degenerative processes associated with aging[1]. It results from an imbalance between the production of reactive oxygen species (ROS), and protective effects (inhibition of free radical production, direct free radical scavenging or detoxification). Free radicals are produced by endogenous (biological metabolism) or exogenous (ionizing radiations, UV light, or pollution) processes. These free radicals can act on DNA, proteins, and lipids, in both polar and nonpolar compartments. Antioxidants are compounds that can prevent formation of free radicals by one (or more) of several mechanisms: (1) scavenging species that initiate peroxidation, (2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (3) quenching $O^2$ − preventing formation of peroxides, (4) breaking the auto oxidative chain reaction, and/or (5)

reducing localized $O^2$ concentrations[2]. The most effective antioxidants are those that interrupt the free radical chain reaction. Usually containing aromatic or phenolic rings, these antioxidants donate H to the free radicals formed during oxidation becoming a radical themselves. These radical intermediates are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of quinone structures[2]. Phytochemicals which are non nutritive, naturally occurring chemical constituents of plants are example of such antioxidants.There are huge evidences concerning the health promoting and disease preventing potential of plant in dietary context and the group of compounds that appear to be key contributors to these potentials are the phytochemicals[3,4]. Phytochemicals, as antioxidants, are highly efficient to scavenge these free radicals and there by inhibit the progression of many degenerative diseases of now-a-days[5].Since plants are major source of phytochemicals, medicinal plants are an important research area for novel and bioactive molecules for drug discovery. Phytochemicals are divided in various subclasses including phenolic acids, lignans, flavonoids, and tannins. They exert wide range of polarity due to their large variety of chemical structure[6,7].Scientific characterisation and isolation of this specific bio potential compound is immensely important for new drug.In this scenario one solvent with specific polarity is unable to dissolve the whole range of bioactive compounds of a plant. Fractionation with solvents of increasing polarity suitable for the primary isolation of phytochemicals.Some natural plants are being used extensively as food and health supplements, which help to combat diseases but due to lack of scientific evidence considerable part of them still remain less focused. In modern civilization where the plant derived drug, life style modification and food based approach in combating the degenerative diseases become the agenda of modern research it is beneficial to fabricate as more as possible the less focused plant extract in medicine[8]. Such type of less focused edible medicinal leafy vegetables are *Typhonium trilobatum* and *Trichosanthes dioica,* commonly known as parwal and kharkol.In a previous work [9,10], it has been shown that it is a potent source of nutrients and phytochemicals. In spite of flurry of researches about use and other parts of these medicinal plants, no scientific documentation on in vitro antioxidant assay of different solvent fraction and their characterisation with thin layer chromatography has been made. So, identification of specific phytochemicals and their antioxidant assay are present concern of the study where *Typhonium trilobatum* and *Trichosanthes dioica,* are selected as the subject. Hopefully this will be helpful for future studies on related aspects.

## Materials and Method:

**1. Identification and authentication of sample**: Whole plant of *Typhonium trilobatum* and *Trichosanthes dioica* were submitted to the Herbarium of Calcutta University, Kolkata. It was indentified (Accession No. 20012 and 20013 respectively) and authenticated by taxonomist of the Calcutta University Herbarium, Kolkata.

**2. Sample Collection:** The plant was collected from different district of West Bengal as well as from different local market.

**3. Sample preparation:** The leaves were shade dried for 3-4 days and grounded into powder and stored for further study.

**4. Preparation of extract**: the samples were shade dried for 3- 4 days and grounded into powder. Powdered leaf of each sample (10g) was mixed with 100ml 80:20 methanol water by maceration for 24 hour. Then the solution was filtered and the filtrates were dried using rotary evaporator under reduced pressure.

**5. Fractionation of extracts:-**

The dried methanol extract was redissolved in 100ml of 80 % aqueous methanol and taken in a separating funnel; 100 ml petroleum ether was added and solution was shaken for few minutes and allowed for phase separation. The organic petroleum ether phase was separated; the lower methanolic phase was collected. Aqueous methanol was taken again in separating funnel; 100 ml of chloroform was added and the funnel was shaken for few minutes and allowed for phase separation. The lower chloroform phase was collected. 10-20 ml of water was added to the aqueous phase and taken in a separating funnel; 100 ml of diethyl ether was added in the separating funnel and shaken for few minutes. Diethyl ether phase was collected and the remaining methanolic fraction was further re-extracted with ethyl acetate and the residual phase is treated as aqueous phase. All the fractions were collected and dried using rotary evaporator and subjected for in vitro antioxidant assay[11]**.**

***6. In vitro*- bioassay:-**

**6. i Determination of total polyphenol content**

To measure the total polyphenol content, Folin–Ciocalteu assay was employed[12]. 0.2 mL of 80% methanolic extract of samples (1mg/ml) was added with 1 mL of Folin–Ciocalteu's phenol reagent (10 fold diluted). 0.8 mL of 2% $Na_2CO_3$ and 60% methanol were added successively. Then the reaction mixture was incubated at room temperature for 30 min and were spectrophotometrically analysed at 740 nm. The calibration curve was plotted using gallic acid (20–100 mg/ml) as standard and the result of polyphenol content was represented as mg of gallic acid equivalent per g of dry extract.

**6. ii Determination of flavonoid content**

Aluminium chloride method[13] was used to quantify total flavonoid content. An aliquot of extracts (0.1ml) or the standard solution of quercetin (20 to 100 mg/ml) was added to a 10 ml volumetric flask containing 4 ml of distilled water. 0.3 ml 5% $NaNO_2$ was added. After 5 min, 0.3 ml 10% $AlCl_3$ was added. At 6th min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The absorbance was measured at510 nm and the results were expressed as mg of quercetin equivalent per g of dry extract.

**6. iii Total antioxidant activity by the phosphomolybdenum method:**

The total antioxidant activities of the plant leaf extract were evaluated by the phosphomolybdenum complex formation method[14] .100-300µg different concentration of each leaf extract were added to test tube containing 3 ml of distilled water and mixed with 1 ml of reagent solution (0.6 M $H_2SO_4$, 28 mM $Na_3PO_4$ and 4 mM ammonium molybdate).The vials were capped and incubated in water bath at 95 °C for 90 minutes. After, cooling, the absorbance was measured at 695 nm against the reagent blank. The antioxidant activity is measured against an ascorbic acid calibration curve.

**6. iv FRAP Assay**

FRAP values were evaluated by the method of Benzie and Strain[15]. To prepare working FRAP reagent, 50 ml of 300 mM acetate buffer (pH-3.6) was mixed with 5 ml of 40 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) dissolved in 40 mMHCl and 5 ml of 20 mM $FeCl_3$. 100 µg of extract was added to 3 ml of freshly prepared working FRAP reagent. The absorbance at 593 nm was measured immediately and after 4 min of incubation at 37 ºC. The change in absorbance was recorded as the final absorbance. For plotting calibration curve, $FeSO_4.7H_2O$ was used as standard at various concentrations (100-500 µM/l). The ferric reducing ability of sample was expressed as FRAP value (µM of $Fe^{2+}$ equivalent).

**6. v. DPPH radical scavenging activity**

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity was evaluated by the method of Hsu et al., 2007 [16] with minor modifications. 3 ml of 0.1 mM DPPH solution was mixed with 1 ml of various concentrations (100 to 300 µg/ml) of leaf extract. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark. The reduction of the DPPH free radical was measured by reading the absorbance at 517 nm by a spectrophotometer against methanol blank. . The solution without any extract and with DPPH and methanol was used as control. The percentage of inhibition DPPH radical was calculated as: [(Absorbance of control – Absorbance of sample)/ Absorbance of control] X 100.

$IC_{50}$ value of each sample was determined from the graph between sample concentration and the percentage of DPPH radical inhibition.

**6. vi Determination of $ABTS^+$ radical scavenging activity:**

For ABTS assay, the method of Dimitrina (2010) and Roberta (1999) with some modifications was followed[17,18]. ABTS was dissolved in water to make a concentration of 7 mmol/L. ABTS+ was produced by reacting the ABTS stock solution with 2.45 mmol/L potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the test of samples, the ABTS+ stock solution was diluted with 80% methanol to an absorbance of 0.70 ± 0.02 at 734 nm. 4.85 ml of diluted ABTS+ was added to

0.15 ml of samples solution of different con. (100-300 µg), and the absorbance was taken 6 min after the initial mixing. BHT (0.1 mg/ml) was used as standard. The activities of the samples were evaluated by comparison with a control (containing 4.85 ml of ABTS solution and 0.15 ml of 80% Methanol). This activity is given as percentage.

ABTS+ scavenging activity (%) = $[(Abs_{control} - Abs_{sample}) / Abs_{control}] X 100$

The results were expressed as $EC_{50}$.

**7.Thin-Layer Chromatography (TLC):-**

Thin layer chromatography (TLC) was used to separate the different constituents present in the extract into different spots on the chromatoplate spotted on silica gel precoated TLC plate and allowed to rise in different solvent systems in saturated TLC chamber. The chromatograms developed on the slide were dried and observed visually (after placing them in iodine chamber) for the various different spots of plant extract. The developing solvent used in extract is Petroleum ether and ethyl acetate with ratio 1: 1. Their $Rf$ values were recorded as the ratio of distance travelled by spots to the distance travelled by solvent system as described by Kajaria et al., 2011. The retention factor was calculated using[19]

$Rf$ = Distance move by the substance (cm) / Distance move by the solvent (cm)

**Statistical Analysis**:

Statistical Analysis was performed with help of SPSS 17. Descriptive statistical analysis was performed to calculate the means with corresponding standard deviation (SD). Also One Way Analysis of variance (ANOVA) followed by post hoc Tukey's test was performed to compare the mean values. $p<0.05$ was taken to be statistically significant.

## Result and discussion:

The results of *Typhonium trilobatum* and *Trichosanthes dioica* were expressed in table 1 and 2 respectively.

**Table 1: In vitro antioxidant activity of five different solvent fractions with increasing polarity of 80% methanol leaf extract of *Typhonium trilobatum.***

| Solvent | Yield % | Polyphenol mg/g extract | Flavonoid mg/g extract | TAC mg/g extract | FRAP Mm Fe(II) equivalent | DPPH $IC_{50}$ value µg | ABTS $IC_{50}$ value µg |
|---|---|---|---|---|---|---|---|
| Petroleum ether | $2.69^{c}$±.22 | $17.16^{b}$±.70 | $21.56^{b}$±.80 | $102.97^{b}$±1.66 | $127.00^{b}$±1.24 | $1542.92^{e}$±21.1 | $765.58^{b}$±3.52 |
| Chlo-roform | $1.41^{b}$±.08 | $12.50^{a}$± .75 | $12.96^{a}$±1.05 | $9.29^{a}$±.28 | $92.99^{a}$±1.98 | $1472.79^{d}$±15.1 | $867.32^{c}$±18.9 |
| Diethyl ether | $0.15^{a}$ ±.02 | $193.56^{e}$ ±1.45 | $122.42^{e}$±1.64 | $268.96^{e}$±1.36 | $211.00^{d}$±5.56 | $142.95^{a}$±7.99 | $62.59^{a}$±2.06 |
| Ethyl acetate | $3.43^{d}$±.20 | $107.79^{c}$±2.48 | $62.62^{c}$±2.15 | $158.48^{c}$±2.60 | $156.70^{c}$±6.56 | $338.42^{c}$±17.91 | $76.13^{a}$±56.6 |
| Aqueous | $9.93^{e}$±.40 | $171.76^{d}$±1.25 | $92.00^{d}$±2.64 | $169.44^{d}$±1.16 | $1006.76^{e}$±5.31 | $233.77^{b}$±5.54 | $83.38^{a}$±.32 |

Values are represented as mean ± SD. Values followed by different superscript letter(s) within each column are significantly different at $p$ <0.05 by Tukey test.

**Table 2: In vitro antioxidant activity of five different solvent fractions with increasing polarity of 80% methanol leaf extract of *Trichosanthes dioica.***

| Solvent | Yield % | Polyphenol mg/g extract | Flavonoid mg/g extract | TAC mg/g extract | FRAP Mm Fe(II) equivalent | DPPH $IC_{50}$ value µg | ABTS $IC_{50}$ value µg |
|---|---|---|---|---|---|---|---|
| Petroleum ether | 0.85±.03 | $28.61^{b}$±2.78 | $43.19^{b}$±2.63 | $207.01^{b}$±2.81 | $149.00^{a}$±11.77 | $1340.67^{d}$±18.34 | $649.72^{c}$±5.26 |
| Chlo-roform | 0.93±0.04 | $19.33^{a}$±0.66 | $15.94^{a}$±1.40 | $14.62^{a}$±0.84 | $146.33^{a}$±3.5 | $1249.67^{c}$±8.50 | $755.32^{d}$±4.28 |
| Diethyl ether | 0.32±0.02 | $236.14^{e}$±5.07 | $417.04^{e}$±4.63 | $427.69^{e}$±6.08 | $510.00^{b}$±11.13 | $99.67^{a}$±1.87 | $52.90^{a}$±3.22 |
| Ethyl acetate | 2.36±.22 | $147.60^{c}$±3.13 | $196.98^{d}$±3.52 | $294.21^{c}$±4.26 | $468.02^{b}$±3.73 | $196.37^{b}$±3.72 | $85.42^{b}$±1.68 |
| Aqueous | 5.80±.3 | $222.82^{d}$±2.77 | $169.24^{c}$±8.05 | $336.85^{d}$±3.99 | $1417.67^{c}$±22.50 | $114.78^{a}$±4.86 | $54.31^{a}$±3.10 |

Values are represented as mean ± SD. Values followed by different superscript letter(s) within each column are significantly different at $p < 0.05$ by Tukey test.

Methanolic crude leaf extract of *Trichosanthes dioica* and *Typhonium trilobatum* were fractionated by different solvent of increasing polarity to separate the bioactive compounds of samples according to their polarity. Crude extract may contain many impurities that will interfere with the result. Fractionation helps to partition the crude extract result in purification to some extent. Since, aqueous methanol is able to dissolved non-polar compounds to some extent in addition to polar compounds; it is used as primary solvent for extraction of the phytochemicals. It was seen that the most potent fraction was diethyl ether followed by aqueous> ethyl acetate> petroleum ether> chloroform for both the samples and *Trichosanthes dioica* was observed to be more potent than *Typhonium trilobatum* in all solvent fractions. Since diethyl ether, ethyl acetate are moderate polar, it is confirmed that the moderate polar compounds of both the sample leaf are most active followed by polar compounds soluble in aqueous fraction.DPPH is stable nitrogen centered free radical and is extensively used for determining antioxidant activity. DPPH assay measures hydrogen (or electron) donating ability of the samples thereby changing DPPH radical from purple to yellow colour and converting it to its reduced form[20]. The results were expressed as $IC_{50}$ value so, lower $IC_{50}$ values indicates better free radical (DPPH) scavenger. Result showed that the samples are able to scavenge DPPH with respect to BHT standard ($IC_{50}$ 28.19±1.80µg).ABTS is also frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods. In this assay, ABTS is converted to its radical cation by addition of potassium persulfate. This radical cation is blue in colour and absorbs light at 734 nm[17,18]. The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and ascorbic acid. During this reaction, the blue ABTS radical cation is converted back to its colourless neutral form. The samples leaf extracts is able to scavenge ABTS radical cation in terms of $EC_{50}$, with respect to BHT ($EC_{50}$75.01±0.50µg).FRAP assay is based on the capability of the sample to reduce the $Fe^{3+}$ to $Fe^{2+}$ in the presence of TPTZ, forming a blue coloured Ferrous - TPTZ complex with an absorption maxima at 593 nm[15]. The mean FRAP values, expressed as Fe (II) (µM)/g of dry extract. Result suggests both the sample is able to reduce the ferric ion to ferrous state.Total antioxidant capacity is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidants and the subsequent formation of a green phosphate Mo (V) complex at acidic pH values. Electron transfer occurs in this assay which depends on the structure of the antioxidant[14]. It is observed that moderate polar diethyl ether fraction of both samples are most active in electron transfer in comparison to ascorbic acid.

The above performed in vitro antioxidant assay and quantitative measurement of phytochemicals suggest that both the sample are quite good in free radical scavenging due to their high amount polyphenol and flavonoid content. But diethyl ether (moderate polar) is the most active fraction followed by aqueous (polar) and ethyl acetate (moderate polar). These findings suggest that majority of bioactive components are extracted in these solvent polarity.

For the identification of metabolites showing antioxidant potentials the fractions are subjected to thin layer chromatography to indentify the specific phytochemicals of them by comparing their R*f* value with the standard R*f*value. Quercetin and gallic acid are used as standard metabolites. From TLC result it is observed that among five different fractions diethyl ether, ethyl acetate and aqueous fraction are active for both sample extracts and the specific phytochemicals present are quercetin and gallic acid.


**Figure 1: Thin layer chromatography of different solvent fractions of *Typhonium trilobatum* 80% methanolic leaf extract**.

Rfvalue of diethyl ether and ethyl acetate are .78 and .72 respectively which compared with Rf value of the standard quercetin (.8) and Rf value of aqueous fraction (0.22) compared with Rf value of gallic acid (0.26).

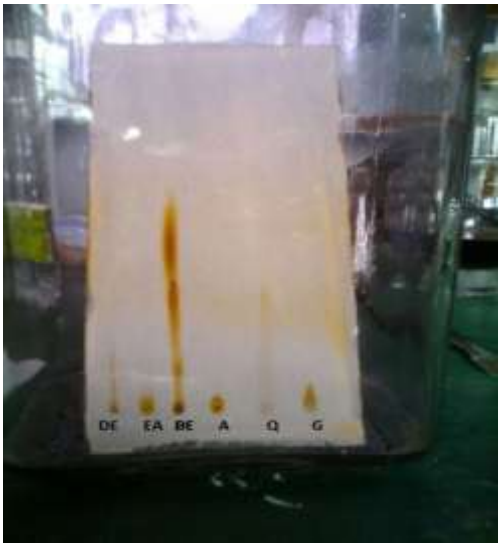


**Figure 2: Thin layer chromatography of different solvent fractions of *Trichosanthesdioica*80% methanolic leaf extract**

It is clearly depicted in the results that the active fractions are diethyl ether and ethyl acetate as their Rf value (0.76 and .7) is compared with Rf value of the standard quercetin (.8) and Rf value of aqueous fraction (0.22) compared with Rf value of gallic acid (0.26). Petroleum ether and chloroform fraction were not considered as active fractions because their radical scavenging potential are very poor in comparison to other active fraction.

## Conclusion:

The present study is an approach to identify the phytochemical constituents and report their antioxidant potential of *Trichosanthes dioica* and *Typhonium trilobatum.* The most revealing feature is that both the samples contain bioactive compounds which as mostly moderate to highly polar. So, the non-polar compounds contribute very littleto disease preventive potential in term of antioxidant activity of the sample. Results of chromatography highlight the presence of specific phytochemicals gallic acid and quercetin. It can be concluded that this phytochemicals are responsible for their antioxidant potential and these compounds are best extracted by moderate polar solvent followed by high polar solvents. Further extensive work on them may lead to a horizon of modern drug design and development.

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