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Effect of solvents on qualitative and quantitative phytochemical constituent profiles of fresh leaves of TV26

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Abstract
The aim of this study was to qualitatively and quantitatively screen and identify major phytochemical groups from leaves extract of TV26 extracted by nine different solvents having different polarities. Qualitative screening suggested acetone, methanol, ethanol and ethyl acetate to be the most potent solvent for various phytochemical extractions like flavonoid, tannin, steroid, diterpenes, terpenoids, coumarin, cardiac glycoside, saponin, protein and reducing sugar. The highest percent of radical scavenging was recorded in cold water extracts (5mg/ml) i.e. 91.10% and was at par with 93.40% percent scavenging activity of ascorbic acid taken as standard (5mg/ml). Methanol, acetone, ethanol and ethyl acetate gave the best results with the total phenol content value (GAE) recorded as 100.60mg/g, 87.07mg/g, 58.73 mg/g and 51.47mg/g respectively with methanol giving the best result. Acetone extracts (5mg/ml) showed higher ferric reducing power with IC_{50} value 426.45±1.12 µg/ml compared to the standard (ascorbic acid) 270.35±0.66 µg/ml. Our findings suggest that the polar solvents were more beneficial and potent against the other non polar counterparts during phytochemical extraction but the polarity of solvents need not be in increasing order since we can assume from our results that acetone being less polar than ethanol, methanol and water showed better results. In addition to different polarities, state of the sample and extraction technique is also crucial for better extraction.

Keywords: TV26, Phytochemicals, Solvent, Qualitative, Quantitative.

Tea, the most popular health beverage next to water in the world, has aroused great interest among the world of scientific research due to its beneficial health effects. It belongs to the genus *Camellia* under Theaceae family with, *Camellia sinensis* (L.)O. Kuntze being the mostly used species for making the health beverage (Kaundun *et al.* 2000). The important phytochemicals in tea includes the polyphenols (catechins and flavonoids), alkaloids (caffeine, theobromine, theophylline etc.), volatile oils, polysaccharides, amino acids, lipids, vitamins (e.g., vitamin C), inorganic elements (e.g., aluminium,fluorine and manganese etc.) with polyphenols being the most important compound of pharmacological importance (Sharangi 2009). It has already been proved in series of experiments that abundant polyphenols in tea imparts many health protecting activities (Manzocco *et al.* 1998). These compounds have a wide range of pharmaceutical properties which includes antioxidative, anticarcinogenic and antiarteriosclerotic (Atoui *et al.* 2005; Dufresne and Farnworth 2001; Filip and Ferraro 2003; Wang and Helliwell 2001). Polyphenolic compounds present in tea may reduce the risk of a variety of illnesses, including cancer, coronary heart disease, atherosclerosis, high blood cholesterol concentrations and high blood pressure. Most of the research work has been focused on manufactured tea or processed tea putting a limitation as such to tea plant (Mukhtar and Ahmad 2000).

Varied range of solvents has been used for extracting polyphenols from plants (Chavan *et al.* 2001) and the role of extracting solvents and the method of extraction on total extraction yield has already been highlighted in several articles (Goli *et al.* 2005). Extraction method should ensure complete extraction of the desired compounds of interest without any chemical modification (Zuo *et*

al. 2002). Extraction and determination of biologically active compounds depends upon the type of solvent used where solvents will diffuse into solid plant tissue and solubilize compound with same polarity (Tiwari *et al.* 2011).

Aqueous mixtures of ethanol, methanol and acetone, water, are commonly exploited to extract plants constituents (Sun and Ho 2005). Researchers have reported use of aqueous methanol, acetone and ethanol (Martinez *et al.* 1997; Wang and Helliwell 2001), absolute methanol (Yao *et al.* 2004), absolute ethanol (Opie *et al.* 1990) and boiling water for the extraction of polyphenols from green, black and mate teas (Turkmen *et al.* 2006). Different solvents like water, aqueous ethanol in different extracting time has been employed to extract phenolics from green and white tea (Rusak *et al.* 2008).

Very little research has been done on phytochemical screening of tea using range of organic solvents. Research has been mostly limited to standard solvents and processed tea thus limiting the study of different phytochemicals in fresh leaves of tea extracted by range of organic solvents. Therefore our study mostly focused on preliminary area of research covering the qualitative and quantitative phytochemical screening as well as studying the antioxidant activity of fresh leaves of TV26 based on extracting solvents of different polarities. The range of organic solvent system with different polarities included absolute hexane, benzene, chloroform, diethylether, ethylacetate, acetone, ethanol, methanol and water.

Materials and methods

Chemicals

10% $FeCl_3$, 1% $FeCl_3$, chloroform, concentrated H_2SO_4, glacial acetic acid, 5% copper acetate, 10%NaOH, Benedict reagent, concentrated HNO_3, distilled water, 2,2-diphenyl-1-picrylhydrazyl, Hexane, Benzene, Diethylether, Ethylacetate, Acetone, Ethanol, Methanol and Water, sodium phosphate buffer (0.2M, pH 6.6), K2 $[Fe(CN)_6]$ 1(% w/v), TCA (10%), $FeCl_3$ (0.1% w/v), Folin reagent, 5% Na_2CO_3, gallic acid, ascorbic acid.

Variety selection

TV26 (Tocklai variety) was chosen as the experimental variety and leaves were collected from tea garden of University of North Bengal. TV26 is a variety of Cambod origin with average quality, high yield and is moderately tolerant to drought (http://www.tocklai.net/activities/tea-cultivation/tra-garden-series-clones/).

Sample extraction

Fresh leaves of TV26 collected were washed thoroughly under running tap water, air dried and then pulverized using a grinder. The sample was weighed and 3g each was distributed equally and immersed in 30 ml each of nine different solvents ranging from polar to non polar. Nine different solvents in increasing order of polarity namely hexane, benzene, chloroform, diethylether, ethylacetate, acetone, ethanol, methanol and water were chosen as extracting solvents. After 48 hours the aqueous cold extracts was centrifuged and the supernatant thus collected was dried and dissolved in methanol. The aliquots of different aqueous extract were thus stored at room temperature.

Qualitative screening of phytochemicals

Qualitative test for phytochemicals included test for flavonoid, tannin, steroid, terpenoid, cardiac glycoside, diterpenes, coumarin, reducing sugar, protein, and saponin with slight modification (Brain and Turner 1975; Kumar *et al.* 2009; Ngbede *et al.* 2008).

Flavonoid

To 250µl of sample few drops of 10% $FeCl_3$ was added to which a blue or green coloration confirmed the presence of flavonoids .

Tanin

Appearance of blue or green colour formation on addition of few drops of 1% $FeCl_3$ to 250 µl of extract confirmed the presence of tannins.

Steroid

About 250 µl of extract was evaporated and dissolved in 2ml of chloroform and about 2ml of concentrated H_2SO_4 was added from the sidewall of the test tube. Appearance of reddish brown colour ring confirmed the presence of steroids.

Terpenoid

The evaporated extract (250 µl) was dissolved in chloroform and concentrated H_2SO_4 was added from the sidewall of test tubes and then shaken.

Formation of red to reddish brown coloration at the base confirmed the presence of terpenoids.

Cardiac glycoside

250 µl of extract was evaporated and to it 1ml of glacial acetic acid, one drop of 10% $FeCl_3$ and 1 ml of concentrated H_2SO_4 was added. A brown ring at the interface indicated the presence of cardiac glycosides.

Diterpenes

Copper acetate was performed to confirm the presence of diterpenes wherein addition of few drops of 5 % copper acetate to 250 µl of extract dissolved in equal volume of distilled water formed emerald green color.

Coumarin

Yellow coloration on addition of about 500 µl of 10% NaOH to 250 µl of sample confirmed the presence of Coumarins.

Reducing sugar

Benedict test was performed to estimate the presence of reducing sugar in the extracts wherein 1ml of Benedict reagent added to 250 µl of sample gave green coloration (color varies from green to red depending upon the percentage of reducing sugar present).

Protein

Xanthoproteic test was performed where 1ml concentrated HNO_3 was added to about 250 µl sample thus giving a yellow precipitate.

Saponin

Froth test was conducted to confirm the presence of saponin with appearance as well as persistence of froth while shaking the sample diluted with distilled water.

Quantitative screening of Phytochemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

As mentioned by (Blois 1958), DPPH was used to determine the antioxidant activity of the mixture of compounds extracted employing different solvents. The decrease in absorbance is marked by the free radical scavenging property of the compound, which donates hydrogen atom and scavenges the unpaired electron of the stable free radical of DPPH. To 100 µl of plant extracts (5mg/ml) prepared from different solvents, 1900 µl of methanol was added and shaken. The mixture was incubated at room temperature for 30 minutes in dark. The absorbance was then recorded at 520 nm using spectrophotometer. Ascorbic acid was taken as a standard.

The total scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging (\%)} = \{A_{control} - A_{sample}/A_{control} \times 100\}$$

Where, $A_{control}$ denotes absorbance of only methanol and DPPH and A_{sample} denotes absorbance of sample dissolved in methanol (Plant extract/ standard) along with DPPH.

Folin-Ciocalteu reagent method for determination of phenol

The total phenolic content of the sample was determined using the Folin – Ciocalteu method (Folin and Ciocalteu 1927) with slight modification. 100 µl samples were taken in a test tube and to it 400 µl of 10 % Folin reagent was added (1ml Folin + 9 ml distilled water). The mixture was incubated in dark for 5 minutes at room temperature followed by addition of 1 ml of 5% Na_2CO_3. After incubating it for 2 hrs in dark at room temperature, absorbance was recorded at 730 nm using spectrophotometer. Gallic acid was taken as a standard and the total phenol content was expressed in mg of GAE per g of extract or GAE mg/g.

Ferric reducing power assay

Ferric reducing power assay was done as per the protocol (Aiyegoro and Okoh 2009) with slight modification. In a test tube 250 µl of leaf extract was taken with addition of 625 µl of sodium phosphate buffer (0.2M, pH 6.6), 625 µl of K2 $[Fe(CN)_6]$ 1(% w/v) and incubated for 20 minutes at 50 °C. The tubes were then cooled and centrifuged at 3000 rpm after addition of 625 µl of TCA (10%). The upper layer of the solution or supernatant (625 µl) was mixed with equal volume of distilled water and 125 µl of $FeCl_3$ (0.1% w/v). The absorbance was finally recorded at 700nm. Higher absorbance value indicated higher reducing power.

Results and Discussion

Acetone, methanol, ethanol and ethyl acetate persistently proved to be the most potent solvent for various phytochemical extractions as it gave

positive result for various test like flavonoid, tannin, steroid, diterpenes, terpenoids, coumarin,cardiac glycoside, saponin, protein and reducing sugar (Table1, Fig. 1). However, extraction of phytochemicals varied in some solvents. Methanol, cold water, ethanol, and ethylacetate proved to be the best solvents for extracting cardiac glycosides. Traces of steroid, diterpenes, reducing sugar, saponin was found in diethylether extracts. Traces of phytochemicals were also seen in less polar solvents like chloroform (steroid and saponin) and benzene (steroid and protein). The nature of extracting solvent plays an important role in extraction of potential compounds of antioxidant activity since the compounds differ in chemical characteristics, polarities and solubilities (Ozarkar 2005). Presence of alkaloids, flavonoids, saponins, terpenoids and phenols were reported in plant extracts of *Camellia sinensis* (purple tea) and the solvents with higher polarity i.e. water, ethanol and acetone were found to extract major phytochemicals groups than non-polar ethyl acetate and chloroform (Geoffrey *et al.* 2014). Methnaol showed better extraction properties than acetone and ethylacetate for extracting few phytochemicals like flavonid, tannin, triterpenes, and lipid and reducing sugar in black packaged tea. Other solvents showed minimum activity (Patil *et al.* 2016). The polar solvents and in some cases even the least polar solvents showed best result in extracting phytochemicals from fresh leaves of TV26 and we could therefore infer that in addition to extraction of samples using solvent with different polarities, extraction time and procedure, the state of sample also matters in phytochemical extraction. The phytochemical constituent slowly degenerates from the time of plucking upto manufacturing. Qualitative screening of phytochemicals is important to the pharmaceutical industry since the presence of a phytochemical of interest may lead to its further isolation, purification and characterization (Ugochukwu and Arukweuche 2013).

Table 1. Qualitative phytochemical screening of TV26 extracted by nine different solvents.

	Hexane	Benzene	Chloroform	Diethylether	Ethylacetate	Acetone	Ethanol	Methanol	Water
Flavonoid	-	-	-	-	++	++++	+	+++	-
Tanin	-	-	-	-	+	++++	++	+++	-
Steroid	-	+	+	++	+++	++++	+++	+++++	+
Terpenoid	-	-	-	-	+	++++	++	+++	-
Cardiac glycoside	-	-	-	-	++	-	++	+++++	+++
Diterpenes	-	-	-	+	++	+++++	+++	++++	-
Coumarin	-	-	-	-	++	++++	++	+++	-
Saponin	-	-	++	-	++	+	+	+++	++
Reducing sugar	-	-	-	+	+	+++	++	++++	-
Protein	-	+	-	+	++	+++	++	++	-

+ Positive test; - negative test; the number of + or - indicates the higher or lower intensity.

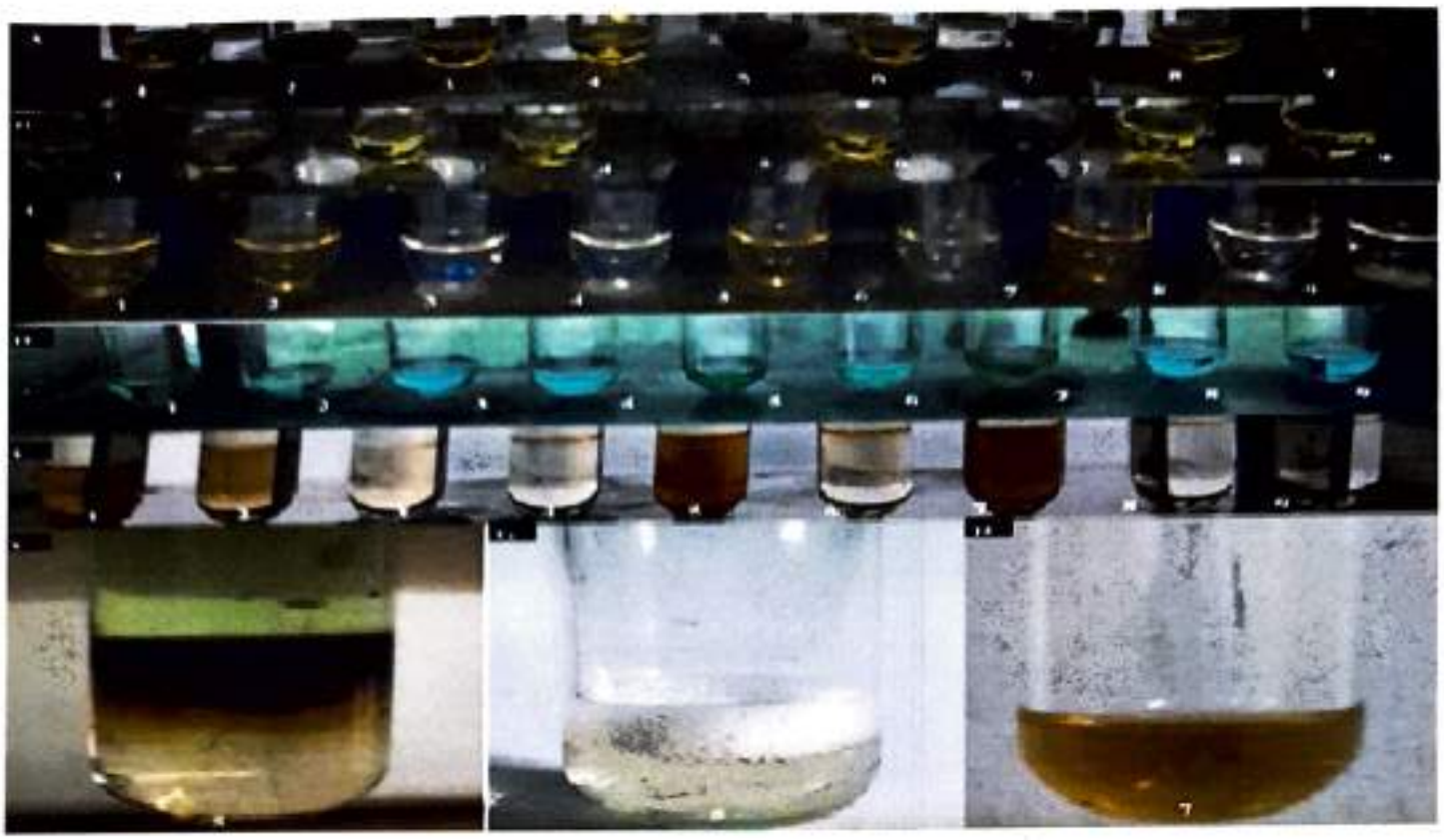

Fig. 1: Qualitative phytochemical screening of TV26 in nine different extracting solvents **1**–Ethanol,**2**–Ethylacetate, **3**-Water, **4**– Benzene, **5**– Methanol, **6**– Diethylether, **7**-Acetone, **8**-Chloroform, **9**-Hexane **[A]** Flavonoid; **[B]** Tanin; **[C]** Coumarin; **[D]** Reducing sugar; **[E]** Terpenoid; **[F]** Cardiac glycoside; **[G]** Saponin; **[H]** Protein.

The antioxidant ability of the sample cannot be concluded only by one method (Patil *et al.* 2016), so we used methods to estimate total phenols quantitatively, DPPH free radical scavenging assay and FRP assay. During DPPH assay, antioxidants acts as a proton donor where the free radical is scavenged and absorbance is decreased thereby rendering change in color from purple to yellow (Liu *et al.* 2010; Manivasagan *et al.* 2015). The highest percent of radical scavenging was recorded in cold water extracts (5mg/ml) i.e. 91.10% and was at par with 93.40% percent scavenging activity of ascorbic acid taken as standard (5mg/ml). Diethylether, ethylacetate, acetone, ethanol, and methanol also gave better results with percent scavenging activity above 50% (Fig. 2). The lowest scavenging activity was seen in hexane extracts. Methanol (Patil *et al.* 2016) ethanol and acetone extract (Turkmen *et al.* 2006b) has already been reported to have strong antioxidant property in black tea. Turkmen (2006) reported varying antioxidant activity with changes in percent concentration of the solvent thus reporting 50% ethanol and 50% acetone with maximum antioxidant potential in mate tea and black tea respectively and also showed the capability of hot water showing moderate antioxidant potential in black tea and higher antioxidant potential in mate tea when compared to other 100% solvent. Thus we could infer that solvent potential may be enhanced or reduced with altering the percent concentration of the solvent.

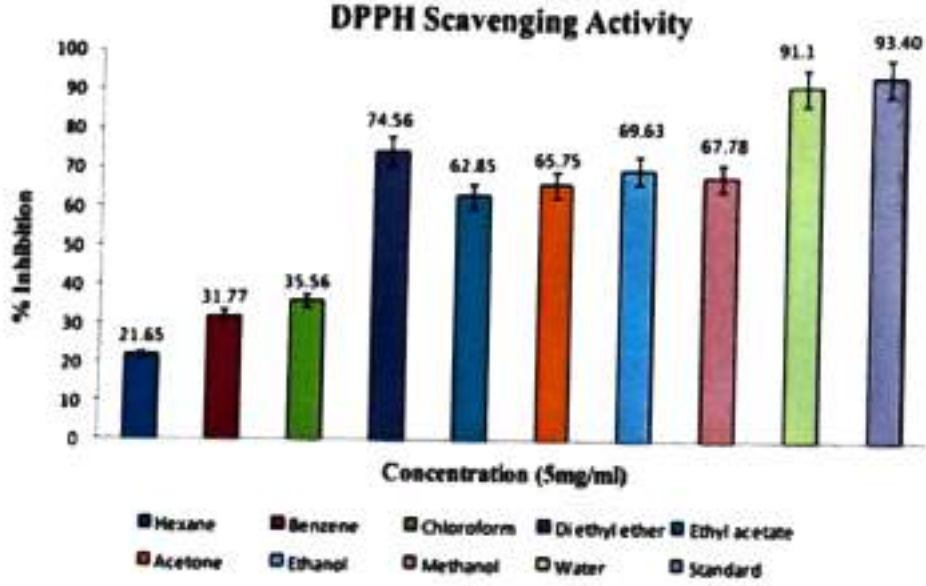

Fig. 2: Percent DPPH free radical scavenging activity of all the nine extracts and ascorbic acid used as standard at concentration 5mg/ml.

The total phenol content was expressed as GAE (mg/g). Methanol, acetone, ethanol and ethyl acetate gave the best results with the total phenol content value recorded as 100.60mg/g, 87.07mg/g, 58.73 mg/g and 51.47mg/g respectively with methanol giving the best result followed by water, diethylether, benzene, chloroform and hexane (Table 2). The lowest value recorded was that of hexane i.e. 4.30mg/g of total phenols. Green tea was found to be a richer source of phenolics than white tea and found 40% aqueous ethanol to be useful for extracting catechin (Rusak *et al.* 2008). Total phenol content estimation following Folin-Ciocalteu reagent method showed 50% acetone, 50% N,N-dimethylformamide (DMF), 50% ethanol and 50% methanol to be suitable for extracting total phenols (Turkmen *et al.* 2006).

Table 2. Determination of total phenolic content (TPC) from fresh leaves of TV26 extracted by different organic solvents.

Solvents	TPC(µg/ml)	GAE (mg/g)	Total phenolic content (mean±SD)
Hexane	2.2	4.3	4.3±0.2
Benzene	3.7	6.9	6.9±0.6
Chloroform	3.0	5.8	5.8±0.2
Diehylether	7.4	12.8	12.8±1.9
Ethylacetate	27.1	51.5	51.5±2.6
Acetone	43.7	87.1	87.1±0.3
Ethanol	31.1	58.7	58.7±3.5
Methanol	54.1	100.6	100.6±7.7
Water	37.5	36.8	36.8±38.2

$y = 0.0075x - 0.0252$; $R^2 = 0.9864$

Acetone extracts showed higher ferric reducing power than other extracts as par with ascorbic acid taken as standard (Fig. 3). The antioxidant compounds acts as reducers causing the reduction of Fe^{3+}/ferricyanide complex to the ferrous form which can be monitored by determining the formation of Perl's Prussian blue at 700nm .The IC_{50} value calculated represents the exact concentration for 0.5 absorbance at 700 nm and the reducing power mostly increases with increasing concentration of antioxidant compounds (Ferreira *et al*. 2007; Manivasagan *et al*. 2015; Patil *et al*. 2016). Acetone extracts showed higher ferric reducing power with IC_{50} value 426.45±1.12 µg/ml compared to the standard (ascorbic acid) 270.35±0.66 µg/ml.

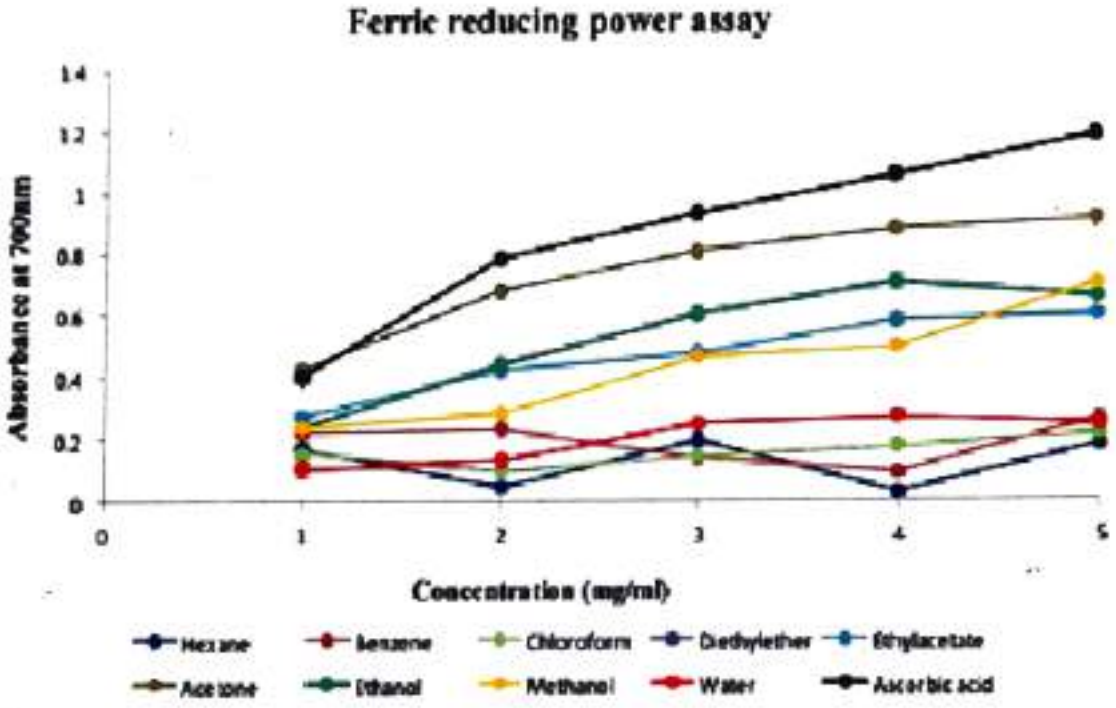

Fig. 3: Ferric reducing power of all the extracts and ascorbic acid (standard) at different concentration (mg/ml).

Conclusion

The qualitative and quantitative phytochemical screening as well as studying the antioxidant activity based on extracting solvents of different polarities was studied for fresh leaves of TV26. The ranges of organic solvent used with differing polarities included absolute hexane, benzene, chloroform diethylether, ethylacetate, acetone, ethanol, methanol and water. Solvents like acetone, methanol, ethanol and ethyl acetate was found to be the most potent solvent for various phytochemical extractions like flavonoid, tannin, steroid, diterpenes, terpenoids, coumarin, cardiac glycoside, saponin, protein and reducing sugar. The highest percent of radical scavenging was recorded in cold water extracts (5mg/ml) i.e. 91.10% and was at par with 93.40% percent scavenging activity of ascorbic acid taken as standard (5mg/ml). Methanol, acetone, ethanol and ethyl acetate gave the best results with the total phenol content value recorded as 100.60mg/g, 87.07mg/g, 58.73 mg/g and 51.47mg/g respectively. Acetone extracts showed higher ferric reducing power with IC_{50} value 426.45±1.12 µg/ml compared to the standard 270.35±0.66 µg/ml. The polar solvents and in some cases even the least polar solvents showed best result in extracting phytochemicals from fresh leaves of TV26 and we could therefore infer that

in addition to extraction of samples using solvent with different polarities, extraction time and procedure, the state of sample also matters in phytochemical extraction.

The collective information of this work could promote future research work in broader ways and to check whether the result varies or remain same among other varieties. Elaborate qualitative phytochemical profiling thus gave an idea about the potency of particular solvent in extracting specific compound of interest.

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