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Antioxidant activity of diosmin, daflon and rutin tablets and extracts of dog rose fruits by permanganate reduction titrimety

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Abstract: Monitoring of permanganate reducing activity (PRA) by titrimetry has been employed to determine antioxidant activity of synthetic diosmin, bioflavonoids and rutin extracted from pharmaceutical tablets, and in aqueous, alcoholic and hydro-alcoholic extracts of dog rose hips and their corresponding lead acetate extracts. The mean PRA as µEq permanganate reduced mg^{-1} diosmin, daflon and rutin have been respectively as 53.8 ± 0.7 (n=16), 45.4 ± 0.6 (n=10) and 109.1 ± 1.4 (n=8) with overall COV about 3.4 per cent. The antioxidant activity with mean PRA in dog rose fruits was mainly found in aqueous, 1.75±0.04 and hydroethanolic,1.67 ±0.02, fractions which was nearly 13 times more than, 0.131±0.007, that due to ethanolic fraction. The titrimetric method offers a simple and inexpensive method for determination of antioxidant potential of plant and drug extracts. It may be useful at least as a primary screen to detect reducing agents for their further evaluation by other sophisticated techniques.
Key-words: Permanganate reduction activity, Titrimetry, Dog rose hips, Diosmin, Daflon, Rutin.

Introduction

The role of flavonoids including diosmin, daflon and rutin as antioxidants in health and disease is well recognized[1, 2]. Similarly numerous therapeutic benefits due to the antioxidants have been attributed to fruits of dog rose[3-5]. Of a large number of available methods to evaluate antioxidants[2, 6], some like. DPPH, FRAP and ABTS have been most often used for demonstrating antioxidant potential in flavonoids[7, 11] and extracts of dog rose[4, 5,12-14]. Cost-effective and simple methods are preferable to complex and advanced procedures as former are well within the operational reach of common laboratories. Acidified potassium permanganate constitutes a strong redox system capable of detecting potential antioxidants. It has been employed as a spot-test for all reducing agents, forming a yellow spot against pink background[15]. The titrimetric assay has been employed for assaying reducing agents such as iron (II) and oxalate; or for determination of reducing potential of otherwise oxidizing agents such as hydrogen peroxide and nitrite[16], and to screen plant extracts for their antioxidant potential[17].Permanganate titrimetry has been employed to assay catalase by measuring consumption of hydrogen peroxide[18] and to determine ascorbic acid in vegetables with advantages of simplicity, fastness and problem-free[19], whereas others have employed more advanced permanganate based methods for determination of ascorbic acid in pharmaceuticals[20-22]. The permanganate method has been employed for determination of the total mass of antioxidant substances and antioxidant capacity per unit mass of serum using redox titration[23]. Rose hips are considered valuable for human consumption because of these serving as an important source of antioxidants such as ascorbic acid, phenolics, flavonoids, carotinoids and tocopherols[3-5, 8-14, 24-26].

In view of foregoing observations, the present work was mooted and experiments designed to optimize a titrimetric method using acidified $KMNO_4$ for determination of antioxidant potential of flavonoids extracted from pharmaceutical tablets, and determine the activity in various extracts of rose hips using a standard antioxidant parameter to quantify antioxidant potential as µEq permanganate reduced per mg test agent.

Experimental

The experiments were carried out at an ambient temperature of 19.0 ± 0.6 ^{0}C. The drugs and chemicals used were of standard purity and quality, obtained from reputed sources in India. The pharmaceutical tablets including Venex-500 (Elder Pharmaceuticals Ltd.,Mumbai), Daflon-500 (Serdia Pharmaceuticals Ltd., Mumbai) and Kerutin-C (Mercury Labs. Pvt. Ltd, Gorwa) were procured from the local market. The pH of test samples was monitored by using pocket pH meter (pH Scan 3, Eutech Instruments, Malaysia), sensitivity 0.01 pH unit.

Reagents and test agents

Potassium permanganate solution:

$KMNO_4$ 800 mg was dissolved in 250 ml water, boiled for 15 to 30 minutes, cooled to room temperature, filtered vide glass-wool, and then standardized against standard oxalic acid 0.05 M (prepared by dissolving 700 mg oxalic acid dihydrate in 111 mL water) by titrimetry while kept warmed to about 70^{o}C as per recommended procedure[16]. The stock solution was frequently checked for normality, and diluted appropriately in water at the time of assay.

Dilute sulfuric acid solution:

10 % (v/v) concentrated sulfuric acid in water.

Standard ascorbic acid solution:

The stock solution made in water, 50 µEq ascorbic acid mL^{-1}, has been standardized against standard iodine solution 0.01 N and also checked against standardized potassium permanganate. The working solution was made in water freshly at the time of use as 1 to 10 µEq mL^{-1} water.

Standard hydrogen peroxide solution:

Working solution of hydrogen peroxide contained 1 to 10 µEq hydrogen peroxide mL^{-1} water. It was made by dilution in water at the time of use from stock solution of 50 µEq hydrogen peroxide mL^{-1} water that has been standardized against potassium permanganate solution. The stock solution was frequently checked every 2 to 3 days for any change in molarity.

Standard ferrous sulfate solution:

The working solution of ferrous sulfate contained iron (II) as 1 to 10 µEq per mL waster in water made by dilution in water from stock solution of 25 iron (II) mL^{-1} water with 0.001M sulfuric. The stock solution has been standardized against standard potassium permanganate.

Lead acetate solution:

A 20 % (w/v) solution of lead acetate was prepared in water and kept well protected by properly stoppered.

Sodium hydroxide solution:

Approximately made 2.5 M in water from sodium hydroxide pellets.

Magnesium chloride solution:

Approximately made as 10 % (w/v) $MgCl_2$ in water.

Dilute standard NaOH solution:

0.1 m NaOH in water.

Neutral ferric chloride solution:

Neutral ferric chloride solution was prepared by dissolving 270 mg $FeCl_3$ in 25 mL water, added few drops of dilute ammonia solution, shaken well and filtered vide Whatman Filter No. 1.

Acid-ethanol solution:

55% (v/v) ethanol in 5 % (v/v) acetic acid in water.

Diosmin and daflon:

Laboratory standards for synthetic diosmin and for daflon were prepared from their respective pharmaceutical tablets: Venex (labeled mass 500 mg synthetic diosmin per tablet) and Daflon (labeled contents 90 % diosmin and 10 % hesperidin) as per standard procedure[27] to the strength of 0.25 % (w/v) in 0.1 M NaOH. The requisite dilutions were made in water to provide flavonoid as 1 mg mL^{-1}.

Rutin:

Rutin was extracted from Kerutin-C tablets (labeled composition 100 mg rutin, 50 mg ascorbic acid, 20 mg menadione sodium bisulfate, 150 mg calcium dibasic phosphate and 300 IU vitamin D_3 per tablet). Finely pulverized mass in pestle and mortar representing 100 mg equivalent rutin was shaken vigorously with about 50 mL water, centrifuged at 5000 rpm for 5 minutes, filtered over Whatman Filter No.1. The residue was thoroughly washed with distilled water till effluent turned colorless, and failed to decolorize acidified permanganate solution, then carefully transferred to volumetric flask, added acid-ethanol to extract rutin[28], allowed standing at room temperature for 15 to 20 minutes followed by centrifugation. The supernatant was filtered along with rinsing with acid-ethanol to make final volume, and saved for the assay. Each mL provided 0.7 mg of rutin. Per cent purity was checked by the methods employed for diosmin and daflon[27].

Samples and preparation of the extracts

Primary extraction:

Fruits of *Rosa canninum* were collected in the month of November from the Institutional premises; 10 g portions of de-seeded material were extracted with 50 mL of solvent with intermittent shaking and left over 24 hours for extraction at 13 ^{0}C ; centrifuged at 6000 rpm for 10 minutes and filtered over Whatman Filter Paper #1. The solvents included water, ethanol and 50% (v/v) ethanol in water. The extracts were saved for antioxidant assay and for further processing wherein each mL represented estimated 200 mg of original plant material. The extracts were checked for pH, and titratable acidity was determined by using 0.5 to 2 mL extract in 5 mL water, added 0.1 mL phenolphthalein indicator and titrated against 0.1M NaOH till pink.

Test for phenolics:

The extracts were taken as 0.1 to 0.2 mL in 5 mL water, added 0.1 mL neutral ferric chloride solution for observation of color change. Positive controls included 0.1 % each of tannic acid, gallic acid, phenol and resorcinol.

Lead acetate extraction:

Twenty mL of each of the filtrates was added 5 mL of lead acetate solution, shaken well and centrifuged at 6000 rpm for 5 minutes and filtered vide Whatman Filter No.1. Sixteen mL of each filtrate was added 1.6 mL of 2.5 M NaOH, shaken well followed by addition of 2.4 mL of 10% $MgCl_2$ solution, again shaken and allowed standing 5 minutes and centrifuged. The filtrate was collected and saved for assay. Each sample extract represented estimated 128 mg of original plant material.

Analytical procedures

Typical direct titrimetry

An aliquot of 5 mL working solution of permanganate was added 1 to 2 mL dilute sulfuric acid, the sample was mixed up and added test extract drop wise till pink color was decolorized and solution turned palish. This was applied to all flavonoids from the pharmaceuticals as well as to the extracts obtained from dose hips. Besides, diosmin extract (n=3) was titrated by incubating 1 mL extract in 4 mL water and 1 mL dilute

sulfuric acid, well mixed and added standard permanganate solution drop wise till the solution acquired a permanent pink coloration to denote end point.

The PRA, µEq permanganate reduced mg^{-1} test material was calculated by using the formula:

$$PRA = (K*N)*(F/M) \quad (1)$$

Where K = mL permanganate utilized; N= Normality of permanganate; M= mass of test material (mg) = ml extract * mg test material mL^{-1}; F=1000, a factor to convert mL normal permanganate into µEq permanganate.

Indirect titimetric assay

This procedure was tested with mainly diosmin and daflon. A 6 mL aliquot of standard permanganate solution was added 2 mL dilute sulfuric acid and 1 mL solvent or test extract. The samples were stoppered and allowed to stand 1 or 2 minutes at room temperature, then titrated for residual permanganate against any standard reducing agent till pink color is disposed off. The aliquot of extract was adjusted by trial experiments so that it did not completely decolorize the permanganate solution during incubation to ensure the samples remain deep pink at the time of titration against standard reductant.

The PRA was calculated by using the following formula while identical volumes of permanganate were employed as titrand in absence and presence of test extract:

$$PRA = (K*N)* [(A-P)/A] * (F/M) \quad (2)$$

Where, A and P are volumes of the titrant used, respectively, in absence and presence of test extract (in mL).

In case of employing reducing agent as titrand in absence and presence of test extract, and using standard permanganate as titrant, the following formula was employed for estimating PRA:

$$PRA = N* (K_p-K_a)* (F/M) \quad (3)$$

Where K_a and K_p are respectively volumes of permanganate in mL used in absence and presence of test extract while using equal volumes of reducing agent.

Results and Discussion

The gross weight of the tablets of Venex-500, Daflon-500 and Kerutin-C were found to be, respectively, in mg as 1032 ± 5 (n=9), 683 ±3 (n=9) and 456 ± 2 (n=10). Each 207 mg Venex powder, 137 mg Daflon powder and 450 mg Kerutin-C powder provided 100 mg equivalent of, respectively, diosmin, mixture of diosmin (90 mg) and hesperidin (10 mg) and rutin. The flavonoids present in the tablets viz., diosmin, hesperidin and rutin are representing three different classes of flavonoids, respectively as, flavone, flavanone and flavonol. The extraction protocol for diosmin and daflon has been previously standardized[27]. For extracting rutin, acid-ethanol used in this study has been recommended by AOAC[28]. With $AlCl_3$ and HCl methods, diosmin and daflon formulations have revealed around 100 per cent purity[27]. Per cent purity of rutin from the tablets was found to be 76.5±0.7 and 80.9 ± 1.4 (n=5 each) respectively with $AlCl_3$ and HCl methods[27]. Lower recovery of rutin is presumably due to loss of rutin on repeated washings with water, required to eliminate interfering ascorbic acid from the Kerutin-C extract.

For direct titrimetry, one is at liberty to use either of the protocols, extract as titrand or as titrant. For indirect assay, it is recommended to use a standard reducing agent as titrant and not as titrand. The results have revealed that while using these as titrand, the PRA values are significantly lower, and erroneous if peroxide, with known oxidizing potential, is employed as titrand. Direct and indirect titrimetry methods were employed for diosmin and daflon to appreciate the comparison of the two approaches. Subsequently, only direct method with extract as titrant was employed for estimating PRA in rutin and dog rose extracts. An overall mean COV with direct and indirect titrimetry has been 3.8 per cent and with direct titrimetry 3.4 per cent.

Permanganate reducing activity of test flavonoids

The results are given in table 1. An overall mean permanganate reducing activity (PRA) for diosmin by direct titrimetry, 53.8 ± 0.7 (n=16), has been about 5 % less than the mean, 56.4 ±1.1 (n=11), found with indirect method ($p<0.05$). Indirect method employing ascorbic acid as titrant provided comparable mean with

respect to direct estimate ($p>0.1$) while iron (II) as titrant has increased estimate by about 10 %. ($p<0.01$). A similar pattern has been observed with daflon extracts. Mean PRA by direct method, 45.4 ±0.6 (n=10), was found to be about 8 % lower than the mean value, 48.9 ±0.7 provided when hydrogen peroxide and iron (II) were employed as titrants (n=12) ($p<0.01$). There was no significant difference between the mean values provided by hydrogen peroxide, 48.5 ±1.6 (n=5) or iron (II), 49.1 ±0.5 (n=7) as titrants ($p>0.1$). An overall increased estimate of PRA with indirect titimetry is possibly due to increased time of contact between extract and acidified permanganate (1-2 minutes), while the contact is quite brief while one is titrating directly. Similarly more activity with diosmin (Venex-500 extract) than with Daflon-500 may suggest that contribution of flavanone, hesperidin, present in daflon to overall PRA is lesser than flavone diosmin.

Table 1: Titrimetric permanganate reduction assay for diosmin, daflon and rutin extracted from tablets

Test material	Method	n	Mean PRA[a]	RSD
Diosmin	Direct	7	54.8±0.8	3.7
	Direct	6	51.3±0.6	3.0
	Direct[b]	3	56.5±0.9	2.8
	Indirect: Iron (II)[c]	6	59.0±0.6	2.6
	Indirect: Ascorbic acid[c]	5	53.2 ±1.0	4.4
Daflon	Direct	10	45.4 ± 0.6	4.0
	Indirect: H_2O_2 [c]	5	48.5±1.6	7.2
	Indirect: Iron (II)[c]	7	49.1±0.5	2.8
	H_2O_2 [d]	9	38.0± 0.9	7.1
	Iron (II)[d]	5	37.4 ± 1.5	9.1
Rutin	Direct	8	109.1 ± 1.4	3.7

n is number of observations; [a] PRA is permanganate reducing activity as µEq permanganate reduced mg^{-1} flavonoid; [b] Extract as titrand; [c] standard iron (II), ascorbic acid or hydrogen peroxide as titrant; [d] Reducing agent as titrand

Compared to diosmin and daflon, the direct estimate of rutin with mean PRA, 109.1±1.4 (n=), regardless of its lower per cent purity (ca.80), has been twice more than diosmin and 2.4 times more than daflon. This is presumably due to presence of catechol moiety and extra hydroxyl function in the rutin which is a flavonol, and flavonoids with catechol moiety and extra hydroxyl function at C-3 are reportedly more antioxidant than others[2].

The results have indicated that indirect method is somewhat better than direct with obvious advantages: (i) a standard time-period of contact is assured between acidified permanganate and test extract; (ii) the end point was sharp and clear; and (iii) the reducing agent need not to be standardized accurately, as is evident from the listed formulae for calculating PRA.

It is not recommended to employ standard reducing agents as titrands. This procedure has provided lower estimates of antioxidant activity. For example, assaying daflon extract in this manner provided mean PRA with iron (II) and hydrogen peroxide, respectively as, 37.4 ±1.5 (n=5) and 39.9 ±0.6 (n=4). The overall mean PRA, 38.0 ± 0.9 (n=9), has been about 22 % lower than the indirect estimate with reducing agent as titrant ($p<0.01$) and 16 % lower than direct estimate ($p<0.01$). Besides, response with diosmin with titrand hydrogen peroxide revealed an increase in mass of diosmin in the incubating sample tended to decrease the mass of permanganate consumed. For instance, at the same mass of hydrogen peroxide (27.7 µEq), the mean PRA at 0.2 mg diosmin was 46.8 ±0.1 which was ca. 14 % more than the value , 40.9 ± 0.8, ($p<0.01$, n=3 each) observed at 0.5 mg diosmin. Similarly at 43.1 µEq H_2O_2 , PRA at 0.5 mg diosmin was found to be 72.5 ± 0.6, which was 18% more than the value observed with 1 mg diosmin, 61.4 ±0.6, ($p<0.01$, n=3 each). Regression analysis relating mass of peroxide µEq per mg diosmin in sample (x-axis) against total mass of permanganate (µEq

consumed) (y-axis) revealed respective and corresponding values as: 43.1 and 61.4; 55.4 and 81.8; 86.2 and 145. This exhibited perfect linearity (r ± se, 0.999±0.001; b±se, 1.96 ±0.05, c, -24.68). At least with hydrogen peroxide as titrand, the permanganate as titrant seems to measure consumption of peroxide by the test extract. This would suggest another possible use of titrimetry in measuring peroxide decomposition by test agents. Lower estimate with iron (II) as titrand may be due to its binding with hydroxyl function of flavonoid and thereby reducing its ability to consume permanganate. The presence of hydroxyl functions in flavonoids is known to have chelating action for metal ions including iron[2].

Permanganate reducing activity of dog rose fruit extracts

Table 2: Organoleptic features and permanganate reducing activity of rose hip extracts by direct titrimetry

Feature	Aqueous extract	Ethanolic extract	Hydro-ethanolic extract
On shaking	High foaming	No foaming	Slight foaming
Color	Orange	Yellowish	Orange-yellow
Marc	Gummy paste	Dark brown hard mass	Light-colored hard mass
Filtration	Sluggish;35 mL in 40 minute	Fast; 44 mL in 10 minutes	Sluggish; 35 mL in 30 minutes
[a]Titrable acidity	0.13 N	0.04 N	0.13 N
pH	3.40	4.00	4.00
[b]Reaction to neutral $FeCl_3$	Light green	Light green	Light green
[c]PRA	1.75±0.04	0.131 ±0.007	1.67 ±0.02
[c]PRA (lead extracts)	0.164 ±0.002	0.621 ±0.009	0.154 ±0.008

[a] Titratable acidity determined with 0.1M NaOH, phenolphthalein as indicator;
[b] For checking presence of phenolics; [c] PRA, permanganate reducing activity as µEq permanganate reduced mg^{-1} plant material; the values are mean ± se of four observations each

The main organoleptic features of the dog rose fruit extracts and their PRA are given in Table 2. Gummy marc with aqueous extraction was apparently due to presence of gums in the fruit pulp[29], and acidic pH and high titratable acidity particular in aqueous and hydro-ethanolic extracts is due to high content of organic acids including citric acid[24, 29] and ascorbic acid[5,13,14,24,25] in the dog rose fruit. As evident, PRA is predominantly found in aqueous and hydro-ethanolic extracts with no difference between the means ($p>0.1$). The mean PRA in ethanolic extract has been about $1/13^{th}$ of either extracts. This implied antioxidant potential of dog rose fruit is attributable to mainly water soluble components such as ascorbic acid and phenolics, and those soluble in ethanol such as flavonoids, tocopherols, caroteinoids and some phenolics, known to be present in dog rose fruits[3-5,12-14,24-26] are contributing to the antioxidant effect minorly. This was further confirmed by performing lead acetate extraction. Both aqueous and hydro-ethanolic fractions lost nearly 90 % of the activity. This is expected because ascorbic acid is known to get removed by lead salts at alkaline pH[30]. Increased PRA, following lead acetate extraction, in ethanolic fraction is presumably due to removal of some interfering constituent from the extract. The simulation experiments have revealed that the lead acetate protocol removed added phenolics like tannic acid, gallic acid, catechol, guaiacol and pyrogallol. The filtrate failed to react to neutral ferric chloride test.. Since dog rose fruit is known to contain significant amount of citric acid, it was desired to test the ability of citric acid to affect permanganate reduction. Addition of citric acid up to 30 mg failed to cause any decolorization of acidified permanganate while a drop of 0.1 % ascorbic acid solution caused complete decolorization of a test aliquot. Similarly, simulation experiments revealed addition of 1 mL of 0.1 % solution of test phenolics significantly decolorized acidified permanganate with order of activity: resorcinol > pyrogallol =phenol >gallic acid ≥tannic acid > ascorbic acid while glucose and oxalate where ineffective at room temperature but effective when incubated in a water bath at about 70 ^{0}C. Thus, free phenolics are quite competent to demonstrate permanganate reducing potential.

All the extracts reacted positively to ferric chloride test producing light green color indicating presence of free or bound phenolics. The positive controls produced deep blue-black coloration (tannic acid, gallic acid), light-green (resorcinol) to distinct green (phenol).

Conclusions

Permanganate titrimetric technique has been found to provide a simple and inexpensive tool to determine antioxidant potential of diosmin, daflon and rutin extracted from the pharmaceutical tablets. The technique demonstrated antioxidant potential in various extracts of the dog rose hips. The permanganate reducing activity in units of μEq permanganate reduced mg^{-1} test material has been found to be approximately within the range of 50 to 60 for diosmin, 45 to 50 daflon, about 109 rutin and 0.13 to 1.75 in extracts of dog rose hips. The technique is anticipated to be employed as a screen in preliminary investigations for screening test agents for antioxidant activities.

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