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Article

Comparative antioxidant and bioavailability studies of Vitamin C in *Phyllanthus emblica* Linn. and its combinations with *Piper nigrum* Linn. and *Zingiber officinale* Roscoe

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Phyllanthus emblica Linn. (amla) is used in Ayurveda, the ancient Indian system of medicine and its major constituent is vitamin C which has effective free radical scavenging property. The purpose of this study was to evaluate the *in vitro* antioxidant activity and the bioavailability profile of vitamin C in amla and its combinations with piperine and ginger in comparison to synthetic vitamin C using New Zealand rabbits. *In vitro* antioxidant activity studies of synthetic vitamin C, amla, amla with piperine and amla with ginger were carried out using different models such as 2,2-Diphenyl-1-picrylhydrazyl, Nitric Oxide, Hydrogen peroxide scavenging methods, Total reductive capability and Oxygen Radical Absorbance Capacity estimation. The study results showed that synthetic vitamin C, amla, amla with piperine and amla with ginger possess significant *in vitro* antioxidant activity. For bioavailability studies, synthetic vitamin C, amla, amla with piperine and amla with ginger 100 mg/kg, were administered orally and the serum samples were analyzed by HPLC at 0, 1, 2, 3, 4, 6, 8, 10, 12 and 24 hours. Bioavailability studies revealed that amla with piperine combination has higher concentration of vitamin C when compared to synthetic vitamin C. This is probably due to presence of piperine, which is a bioavailability enhancer. The present study supports the fact that amla with piperine combination can be an alternative to synthetic vitamin C.

Uniterms: *Phyllanthus emblica* Linn. Vitamin C/antioxidant activity/*in vitro* study. Vitamin C/bioavailability. Vitamin C/synthetic/antioxidant activity. Ginger. Piperine.

Phyllanthus emblica Linn. (amla) é utilizada na medicina Ayurveda, medicina da Índia antiga e seu principal constituinte é a vitamina C, que possui propriedade sequestrante de radicais livres. O propósito deste estudo foi avaliar a atividade antioxidante *in vitro* e o perfil de biodisponibilidade da vitamina C na amla e suas combinações com piperina e gengibre em comparação com a vitamina C sintética, utilizando coelhos da Nova Zelândia. Os estudos de atividade antioxidante *in vitro* de vitamina C sintética, amla, amla com piperina e amla com gengibre foram realizados utilizando-se diferentes modelos para sequestrantes, como 2,2-difenil-1-picrilidrazil, óxido nítrico, peróxido de hidrogênio, capacidade redutiva total e a estimativa da capacidade de absorvância do radical oxigênio. Os resultados do estudo mostraram que vitamina C sintética, amla, amla com piperina e amla com gengibre possuem atividade antioxidante *in vitro* significativa. Para os estudos de biodisponibilidade, administraram-se oralmente vitamina C sintética, amla, amla com piperina e amla com gengibre 100 mg/kg e as amostras de soro foram analisadas por CLAE em 0, 1, 2, 3, 4, 6, 8, 10, 12 e 24 horas. Os estudos de biodisponibilidade revelaram que a associação de amla com piperina tem maior concentração de vitamina C, quando comparada com a vitamina C sintética. Este efeito é provavelmente devido à presença de piperina, que é intensificador de biodisponibilidade. O presente estudo apoia o fato de que a associação de amla e piperina pode ser uma alternativa para a vitamina C sintética.

Unitermos: *Phyllanthus emblica* Linn. Vitamina C/atividade antioxidante/estudo *in vitro*. Vitamina C/biodisponibilidade. Vitamina C/sintética/atividade antioxidante. Gengibre. Piperina.

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INTRODUCTION

Cell damage caused by free radicals is a major contributor to ageing and to degenerative diseases such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction (Percival, 1998).

One line of defense against free radical damage is the presence of antioxidants. Antioxidant means "against oxidation". An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. Some such antioxidants, including glutathione, ubiquinol and uric acid are produced during normal metabolism in the body, other lighter antioxidants are found in the diet and the best known are vitamin E, vitamin C and the carotenoids.

Vitamin C (ascorbic acid) is a water soluble organic compound involved in many biological processes (Gazdik *et al*., 2008). It is one of the most ubiquitous vitamins ever discovered and plays a paramount role as an antioxidant and a free radical scavenger, able to moderate the oxidative stress effects of various diseases (Karslen *et al*., 2005). It has been found in fruits and vegetables like citrus fruits, pepper, cabbage, spinach, strawberries, tomatoes, turnip and other leafy vegetables. The estimated average requirement and recommended dietary allowance of ascorbic acid are 100 mg and 120 mg per day respectively.

Ascorbic acid helps in the metabolism of cholesterol, contributes to the synthesis of the amino acid, protects the DNA of cell from damage and acts as a potential scavenger of free radicals.

Phyllanthus emblica Linn. (amla) has been used in Ayurveda and its major constituent is vitamin C which has effective free radical scavenging property (Khopde *et al*., 2001). The petroleum extract of *Piper nigrum* Linn. (P) (Black pepper) has been reported to have antioxidant activity (Singh *et al*., 2008). *Zingiber officinale* Roscoe(G) (Ginger) has high content of antioxidants which makes it a free radical scavenger (Kikuzaki, Nakatani, 1993; Kikuzaki *et al.*, 1994). Hence, the present study was carried out to evaluate the *in vitro* antioxidant activity of amla and its combinations with piperine and ginger when compared with synthetic vitamin C.

Many analytical techniques including sensors and biosensors have been suggested for detection of ascorbic acid in varied types of samples. HPLC combined with UV-visible detector is the most common method for identification of antioxidant vitamins in biological fluids (Zhao *et al*., 2004). The accepted gold standard method of measuring vitamin C in serum or plasma is high performance liquid chromatography (HPLC) (Emadi-Konjin *et al*., 2005).

Since there are no reports on the bioavailability studies of the combinations of amla with piperine and ginger, this study proposes to investigate the same in rabbits.

MATERIAL AND METHODS

Material

Phosphoric acid (HPLC grade) was obtained from Spectrochem Pvt. Ltd., Mumbai. Methanol (HPLC grade) and HPLC water were obtained from Central Drug House Ltd., Gujarat. Monobasic potassium phosphate (Sd fine-chem Limited, Mumbai) and perchloric acid (Merck specialities. Pvt. Ltd., Mumbai) were used in the study. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), fluorescein, 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) and trolox were obtained from Sigma-Aldrich Inc., USA. Potassium ferricyanide, trichloroacetic acid, ferric chloride, sodium nitroprusside, sulphanilamide, *N*-(1-naphthyl) ethylene diamine dihydrochloride, potassium dihydrogen phosphate, sodium hydroxide, sodium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Sd fine-chem Limited, Mumbai, India. O–phosphoric acid was obtained from Ranbaxy Fine Chemicals Limited, Mumbai. Hydrogen peroxide was obtained from V.L. Products, Mumbai.

Amla (A), piperine (P) and ginger (G) were dry aqueous, alcoholic and hydroalcoholic extracts respectively. All samples including synthetic vitamin C were obtained as gift samples from M/s Green Chem Herbal Extracts and Formulations, Domlur, Bengaluru. Amla with piperine (A+P) was a mixture of amla (99.8 g) and piperine (0.2 g). Amla with ginger (A+G) was a mixture of amla (95.5 g) and ginger (4.5 g).

Animals

New Zealand rabbits of either sex with a body weight of approximately 2 kg were procured from registered breeder M/s Shri Venkateshwara Enterprises, Bengaluru. Animals were housed in animal house facility of KLE University's College of Pharmacy, Bengaluru. All the animals were housed according to CPSCEA guidelines under standard animal house conditions. All the animals were maintained in hygienic conditions with food and water *ad libitum*. All animals were acclimatized to laboratory condition for a week before commencement

of experiment. The study was approved by Institutional Animal Ethics Committee (Reg. No.626/02/a/CPCSEA).

In vitro studies of antioxidant activity

DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity

The free radical scavenging activity can be measured using 2,2-diphenyl-1-picrylhydrazyl by the method of McCune and Johns (McCune, Johns, 2002). 0.1 mM solution of DPPH in methanol was prepared, 1 mL of this solution was mixed with 1 mL of solution of test extract/ standard antioxidant and 1 mL of methanol at different concentrations in the range of 15-75 µg/mL. The mixture was incubated for 10 min in dark. After 10 min, absorbance of the mixture was measured at 517 nm using Ultraviolet-Visible Spectrophotometer (Shimadzu UV-1700 PC spectrophotometer).

The % scavenging activity was calculated using the following equation:

$$\% \text{ SA} = (A_0 - A_1 / A_0) \times 100$$

where % SA= percentage scavenging activity, A_0 = absorbance of control, A_1 = absorbance of sample/ standard.

Scavenging of hydrogen peroxide

The free radical scavenging activity was determined by using hydrogen peroxide (Ruch *et al*., 1989). Different concentrations of the extract and standard in the range 2-10 µg/mL were prepared in distilled water and 0.6 mL of hydrogen peroxide solution (40 mM) prepared in phosphate buffer (pH 7.4) was added to make a final volume of 4 mL. Absorbance of hydrogen peroxide at 230 nm was measured after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and the percentage scavenging activity was calculated.

Total reduction capability

Total reduction capability was estimated using the method of Gulcin (Gulcin *et al*., 2005). Different concentrations of test/standard antioxidant (15-75 µg/mL) in 1 mL of distilled water was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide (1%). The mixtures were incubated at 50°C for 20 min. 2.5 mL trichloroacetic acid (10 %) was added to the mixture and was centrifuged for 10 minutes at 1000 × g. 2.5 mL of upper layer was mixed with 2.5 mL distilled water and 0.5 mL ferric chloride (0.1%) and the absorbance was measured at 700 nm using UV-Visible spectrophotometer (Shimadzu UV-1700). Higher absorbance of the reaction mixture indicates greater reducing power.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined according to Sumanont *et al*. (2004). Nitric oxide radicals were generated from sodium nitroprusside solution in phosphate buffer saline (PBS) at physiological pH (7.4). Sodium nitroprusside solution (100 mM, 0.2 mL), with 1 mL of test/standard antioxidant solution and 1.8 mL of PBS was mixed in different concentrations (2-10 µg/mL). The mixture was incubated at 25°C for 180 minutes. 1 mL of incubated solution was mixed with 1 mL of Griess reagent (Equal portions of 1% sulphanilamide and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride in 2% H_3PO_4). Absorbance was measured at 540 nm using UV visible spectrophotometer (Shimadzu UV-1700) and the percentage inhibition was calculated.

Oxygen Radical Absorbance Capacity assay

The Oxygen Radical Absorption Capacity (ORAC) assay is a method which measures the loss of fluorescein fluorescence over time due to peroxyl-radical formation by the breakdown of AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride). Trolox [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog serves as a positive control inhibiting fluorescein decay in a dose dependent manner (DeLang, Glazer, 1989; Cao *et al*., 1993).

Bioavailability studies

Experimental design and treatment

All the extracts were suspended in water and administered orally to overnight fasted animals in the dose of 100 mg/kg body weight, selected on the basis of acute toxicity studies (OECD guidelines). Bioavailability studies were carried out in 16 rabbits, divided into four groups. Group 1: ascorbic acid, Group 2: amla, Group 3: amla and piperine, Group 4: amla and ginger.

Instrumentation

The HPLC instrument used consisted of Merck Hitachi LaChrom chromatographic system equipped with Hitachi pump L-7110, Rheodyne universal injector 7725 and L-7400 Hitachi UV-visible detector. The chromatographic studies were performed using Thermo scientific ODS hypersil 5 µm, 250 × 4.6 mm i.d. column, at ambient temperature. The mobile phase consisted of 30 mM monobasic potassium phosphate (pH 3.6) and methanol in the ratio 82.5:17.5 (v/v) and the flow rate was

1 mL/min. Chromatograms were recorded at 250 nm and the injection volume was 20 µL.

Sample collection

The bioavailability studies were done by drawing blood samples without the addition of anticoagulant from rabbit's marginal ear vein at 0, 1, 2, 3, 4, 6, 8, 10, 12, 24 h. Serum was separated by centrifugation at 8500 rpm for 10 min and estimation of vitamin C was done by using HPLC (Ghosh *et al*., 2009). Perchloric acid (25 mL of 0.1 M) and 55 mL of distilled water were added to a 20 mL aliquot portion of serum. Addition of acid was needed to maintain the stability of ascorbic acid (Karatepe, 2004).

Standard solution preparation

The stock solution of SVC was 100 µg/mL in mobile phase and all dilutions subsequently were made in mobile phase. The serum was spiked with standard solution of vitamin C to confirm the peak (Scartezzini *et al*., 2006).

Calibration curves

Standard solutions of SVC in the concentration range 0.5-25 µg/mL were prepared and injected into the HPLC system. The analyte peak area values were plotted against the corresponding concentrations of the analyte and the calibration curve was constructed by means of the least square method.

Sample analysis

An aliquot of the sample was injected into the HPLC system in triplicate. The area of SVC peaks obtained after injecting the extract into the HPLC was interpolated on the calibration curve.

Statistical analysis

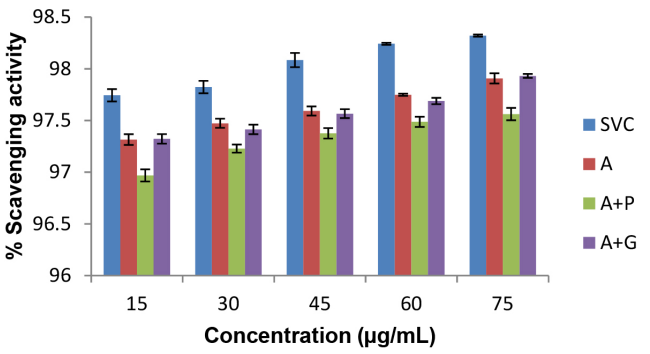
Data were expressed as mean ± SEM. Statistical differences between means were determined by One-way ANOVA followed by Dunnett's post hoc test. Values of $p < 0.05$ were considered as significant.

RESULTS

In vitro antioxidant activity studies

DPPH radical scavenging activity

All the test compounds (SVC, A, A+P, A+G) produced DPPH scavenging activity in the concentration range of 15-75 µg/mL and it was found to increase with increase in concentration (Figure 1). The scavenging effect was found to be decreasing in the order of SVC > A+G > A > A+P at the concentration of 75 µg/mL. There was significant free radical scavenging produced by A ($p < 0.05$), A+P ($p < 0.001$), A+G ($p < 0.05$) combinations compared to SVC. The IC_{50} values of SVC, A, A+P, A+G were found to be 38.14 ± 0.01, 38.30 ± 0.03, 38.44 ± 0.04 and 38.29 ± 0.01 respectively (Table I).



All values are Mean ± SEM (n=3)

FIGURE 1 - Free radical scavenging activity of different concentrations of SVC, A, A+P, A+G by DPPH method.

Hydrogen Peroxide scavenging activity

The hydrogen peroxide scavenging activity of all samples and standard are summarized in Figure 2. The activity was found to decrease in order of A+P > A > SVC > A+G at a concentration of 2 µg/mL. A+P combination produced maximum scavenging of hydrogen peroxide when compared to SVC. The IC_{50} values of SVC, A, A+P, A+G were found to be 1.16 ± 0.01, 1.15 ± 0.01, 1.13 ± 0.001 and 1.17 ± 0.01 respectively (Table I).

Total reduction capability

It was found that the total reduction capability increased with increase in concentration from 15-75 µg/mL for all samples tested but none of the samples showed significant scavenging activity in this method.

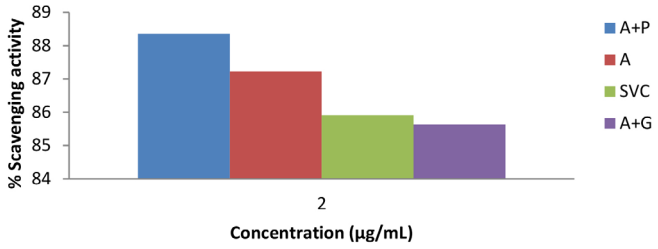
Nitric Oxide scavenging activity

Effect of nitric oxide scavenging activity was found to decrease in the order SVC > A+G > A+P > A at a concentration of 10 µg/mL. Capability to scavenge nitric oxide was found to be concentration dependant at all concentrations from 2-10 µg/mL. Maximum inhibition was produced at concentration 10 µg/mL and the results are summarized in Figure 3. There was a significant scavenging activity produced by A and A+P when compared to SVC ($p < 0.001$) and the IC_{50} values for SVC, A, A+P, A+G were found to be 6.35 ± 0.08, 7.24 ± 0.36, 6.76 ± 0.25 and 6.49 ± 0.42, respectively, as shown in Table I.

Determination of antioxidant capacity of samples

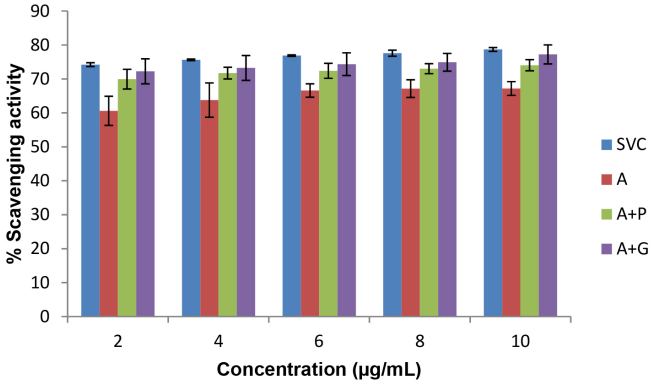
TABLE I - IC_{50} values of SVC, A, A+P, A+G in hydrogen peroxide, DPPH and nitric oxide. All values are Mean ± SEM (n=3)

	IC_{50} values ± SEM (µg/mL) for free radical scavenging activity		
Test/ standard group	**Hydrogen peroxide**	**DPPH**	**Nitric oxide**
SVC	1.16 ± 0.01	38.14 ± 0.01	6.35 ± 0.08
A	1.15 ± 0.01	38.30 ± 0.03	7.24 ± 0.36
A+P	1.13 ± 0.001	38.44 ± 0.04	6.76 ± 0.25
A+G	1.17 ± 0.01	38.29 ± 0.01	6.49 ± 0.42



All values are Mean ± SEM (n=3)

FIGURE 2 - Hydrogen peroxide scavenging activity.



All values are Mean ± SEM (n=3)

FIGURE 3 - Free radical scavenging activity of different concentrations of SVC, A, A+P, A+G by nitric oxide radical scavenging method.

by Oxygen Radical Absorbance Capacity (ORAC) assay

The antioxidant capacity of these compounds was as follows: A+P >A> SVC >A+G .

TABLE II - Antioxidant capacity of SVC, A, A+P and A+G by ORAC Assay

Sl. No	Sample Name	ORAC value (TE/g)
1	SVC	2465
2	A	2651
3	A+P	3150
4	A+G	1875

Bioavailability studies

A good linearity was found from 0.5 to 25 µg/mL of SVC and the linear regression equation was y = 25155x - 15522 (r= 0.9975), where y is the peak area and x is the concentration of Vitamin C expressed as µg/mL (Figure 4). The HPLC method was validated and data shown in Table III.

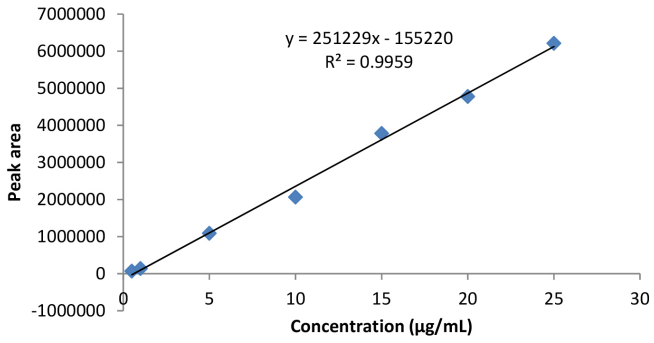


FIGURE 4 - Calibration curve of Vitamin C.

The presence of vitamin C was detected at 3.31 min. Comparisons were made between the standard and sample chromatograms. Larger area indicates larger amount of vitamin C. Little interferences were detected in the chromatogram due to contaminants. By comparing the AUC, higher bioavailability was observed for A+P, followed by A, SVC and A+G (Table IV).

DISCUSSION

The amla fruit contains more than 80% water. It also has protein, carbohydrate, fibre, minerals and vitamins. It also contains gallic acid which is a potent polyphenol. Amla restores the vitality and rejuvenates all bodily systems. It is a rich source of vitamin C and has been used as a powerful antioxidant agent which also boosts immunity.

Vitamin C is important for human beings as it is necessary for the synthesis of intercellular cement "collagen".

TABLE III - Analytical parameters of the HPLC procedure for Vitamin C quantitation

Parameter	**Vitamin C**		
Linearity range	0.5 to 25 µg/mL		
Regression equation	y = 25155x - 15522		
Correlation coefficient (r)	0.9975		
Analyte Concentration (µg/mL)	**5**	**15**	**25**
Repeatability (R.S.D%)[a] (Intra-day precision)	1.89	0.78	1.01
Intermediate precision (R.S.D%)[a] (Inter-day precision)	1.96	0.97	1.13
Limit of Detection (LOD)	0.03µg/mL		
Limit of Quantification (LOQ)	0.1µg/mL		

[a]n = 3

TABLE IV - Results of Bioavailability studies

Pharmacokinetic parameter	**SVC**	**A**	**A+P**	**A+G**
AUC	131.8	1218	2630	79.09
t_{max} (h)	10	12	10	1
C_{max} (µg/mL)	79.794	226.989	465.880	48.537

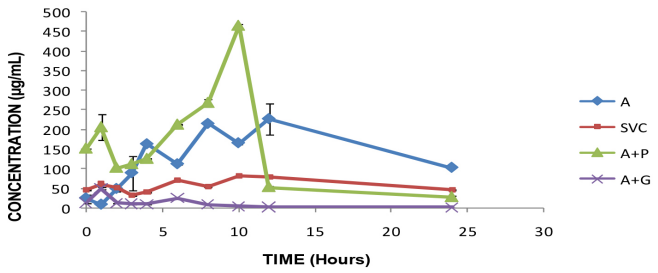


FIGURE 5 - Concentration of vitamin C vs Time plot in SVC, A, A+P, A+G.

Collagen is responsible for keeping the cells of the body together. Hence, vitamin C helps to preserve the normal immune function and promotes rejuvenation of cells.

Recent reports indicate that increased dietary intake of antioxidant-rich foods decreases the incidence of human diseases. However, synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) that have been widely used as antioxidants in the food industry may be responsible for liver damage and carcinogenesis. For this reason, the use of natural antioxidants with lesser side effects, are preferred. This work focuses on the antioxidant activity of selected natural product, amla, alone and in combination with piperine and ginger, compared with synthetic vitamin C for their beneficial antioxidant potential. Chemical investigations have indicated that amla is rich in tannins, alkaloids, phenolic compounds, aminoacids, carbohydrates, vitamin C, quercetin and chebulagic acid (Khan, 2009). *Piper nigrum* is called the king of spices and is one of the oldest spices which contains volatile oil, crystalline alkaloids, piperine, piperidine, piperitine, piperolein A, piperolein B and resins (Manoj *et al*., 2004). The reported chemical constituents of *Zingiber officinale* are cineole, geraniol, citralgingerols, vitamins like thiamine and vitamin C (Kalpagam *et al*., 2003).

Hydrogen peroxide initiates lipid peroxidation weakly. However, it is able to produce active oxygen species by generating highly reactive hydroxyl radical through the Fenton reaction (Powers, Jackson, 2008). All the test samples (SVC, A, A+P, A+G) were significantly different in terms of antioxidant potency. A+P showed the highest scavenging activity which may be due to the terpenoids which are powerful compounds with enormous ability to mop up cell or damage free radicals followed by A, SVC and A+G.

The DPPH radical is a lipophilic and relatively stable nitrogen centred free radical that can accept an electron to become a stable diamagnetic molecule (Yoganandam *et al*., 2010; Bharathi *et al.*, 2010). The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability where DPPH radical serves as the oxidizing substrate which can be reduced by an antioxidant compound to its hydrazine derivative. From the results it is evident that the test compounds are acting as hydrogen donors and A+G combination possesses highest DPPH radical scavenging activity when compared to other

samples which may be due to the presence of gingerol in ginger, one of the polyphenols and vitamin C in amla as its active principle (Kishk, Sheshetawy, 2010).

Oxygen reacts with the excess NO to generate free radicals, nitrite and peroxy nitrite anions (Marcocci *et al*., 1994) and quenching of these free radicals measures the antioxidant potential of a test compound. As A+G combination showed radical scavenging activity which was comparable with SVC, this combination can be an alternative to SVC.

The evaluation of reducing capability is based on the principle that, increase in the absorbance of the reaction mixture by the sample/standard increases the reductive capability (Koksal *et al*., 2011). Owing to their reducing capabilities, antioxidant compounds cause the reduction of ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form. Prussian blue colored complex is formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. All the test compounds under study were found to increase the absorbance in a concentration dependent manner, but none were found to have significant reducing capability.

The ORAC assay has become a valuable and popular method to determine the potential antioxidant activities of various compounds and biological samples because it measures the scavenging capacity against peroxyl radicals which are one of the most common reactive oxygen species in the body. This method is superior to other methods for two reasons. First, the ORAC assay system uses an area-under-curve (AUC) technique thereby combining into a single quantity both inhibition time and inhibition degree of free radical action by an antioxidant. Second, different free radical generators or oxidants can be used in the ORAC assay (Cao *et al*., 1997).

ORAC is a fluorescence method using AAPH which produces peroxyl radicals by undergoing spontaneous decomposition. This method is more sensitive than the spectrophotometric assay as it requires a much lower final standard concentration than the spectrophotometric assay (Cao, Prior, 1998).

Our aim was to compare SVC with A and its combinations for their antioxidant potential and we found that A+P combination showed highest ORAC value followed by A, SVC and last was A+G. This may be due to *in vitro* antioxidant activity of piperine (Mittal, Gupta, 2000).

Since, the second objective of our study was to evaluate plasma concentration of vitamin C in different combinations of amla, it was necessary to estimate the antioxidant potential of all compounds under study like SVC, A, A+P and A+G. As the *in vitro* antioxidant studies revealed that A+P combination showed maximum antioxidant potential, it was desirable to confirm the same by *in vivo* studies with HPLC estimation of serum samples for vitamin C in rabbits.

Numerous assays for ascorbic acid have been employed and they can be divided into three categories - enzymatic, spectrophotometric and chromatographic assays. Enzymatic and spectrophotometric assays are often influenced by interferences leading to overestimation of ascorbic acid in biological samples, and the necessity of modern high performance liquid chromatographic (HPLC) methods for the determination of vitamin C in biological samples have been established (Mittal, Gupta, 2000).

Vitamin C is highly sensitive to factors such as light, heat and pH. A slight change in the mobile phase, solvents and temperature during detection can give false result that would lead to change in retention time. Further the differences in solvent refractive index cause an unstable chromatographic baseline. Selected solvents such as methanol and monobasic potassium phosphate were used as they were found to give best results for the estimation of vitamin C (Hanachi, Golkho, 2009).

In the present study, HPLC estimation of vitamin C revealed that A+P combination has the maximum bioavailability compared to other samples tested (Fig. 5, Table IV). This could be due to the presence of piperine which is used as a bioavailability enhancer and contributing for the increased vitamin C concentration shown by the combination A+P (Gohil, Mehta, 2009). Since piperine enhances the bioavailability of vitamin C present in amla, when used in combination, this combination may be suggested as the best source of vitamin C supplement.

To conclude, antioxidant potential of A+P combination was confirmed to be the best when compared to amla alone and SVC as revealed by *in vivo* studies in rabbits.

CONCLUSION

When compared with the other combinations tested, the A+P combination exhibited the highest concentration of vitamin C both *in vivo* and *in vitro*. This may be due to presence of piperine in pepper which enhances the bioavailability of vitamin C from amla and can be an alternative to synthetic vitamin C.

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