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DETERMINATION OF VITAMIN C CONTENT OF SOME INDIGENOUS FRUITS OF KERALA AND ITS INFLUENCE ON BIOLOGICAL ACTIVITIES

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Abstract

Diets high in fruits and vegetables are widely recommended for their health-promoting properties. Fruits and vegetables have historically held a place in dietary guidance because of their concentrations of vitamins, especially vitamins C and A; minerals, especially electrolytes; and more recently phytochemicals, especially antioxidants. Some indigenous fruits of Kerala like *Phyllanthus emblica* (Gooseberry), *Phyllanthus acidus* (Malay gooseberry), *Averrhoa bilimbi* (Bilimbi), *Syzygium samarangenese* (Rose apple) were selected and their Vitamin c content was analysed in Fresh, Brined and cooked extracts.The fresh extract of *Phyllanthus emblica* (Gooseberry) showed a maximum vitamin C content. The fresh extract of *Phyllanthus emblica* (Gooseberry) on screening for phytochemicals showed the presence of phenols, flavanoids, tannins and alkaloids. The extract also showed a significant anti-lipid peroxidation, anti-inflammatory, anti-diabetic and antibacterial activity.

Keywords: Vitamin C, Fruits, anti-lipid peroxidation, anti-inflammatory, anti-diabetic, anti-bacterial activity.

Introduction

Fruits and vegetables are universally promoted as healthy. Fruits and vegetables include a diverse group of plant foods that vary greatly in content of energy and nutrients. Additionally, fruits and vegetables supply dietary fiber, and fiber intake is linked to lower incidence of cardiovascular disease and obesity. Fruits and vegetables also supply vitamins and minerals to the diet and are sources of phytochemicals that function as antioxidants, phytoestrogens, and anti-inflammatory agents and through other protective mechanisms. Diets high in fruits and vegetables are widely recommended for their health-promoting properties. Fruits and vegetables have historically held a place in dietary guidance because of their concentrations of vitamins, especially vitamins C and A; minerals, especially electrolytes; and more recently phytochemicals, especially antioxidants. Additionally, fruits and vegetables are recommended as a source of dietary fiber (Slavin and Lloyd, 2012). Fruits are also recommended as a source of vitamin C and potassium.

Vitamin C belongs to the water-soluble class of vitamins. Ascorbic acid (AA) is an odorless, white solid having the chemical formula $C_6H_8O_6$. This vitamin is easily oxidized to form dehydroascorbic acid (DHAA), and thus oxidation is readily reversible from DHAA (Groff et al., 1995). The importance of vitamin C was first discovered in 1747. It is the major water-soluble antioxidant within the body. Humans are one of the few species who lack the enzyme to convert glucose to vitamin C. The vitamin readily donates electrons to break the chain reaction of lipid peroxidation. The water-soluble properties of vitamin C allow for the quenching of free radicals before they reach the cellular membrane. Vitamin C is important in collagen formation, thereby resulting in stabilization of the peptide. Indirectly, AA plays important regulatory roles through- out the entire body due to its involvement in the synthesis of hormones, hormone-releasing factors, and neurotransmitters (Groff et al., 1995; Jacob and Sotoudeh, 2002).

Vitamin C is essential for the biosynthesis of collagen, L-carnitine, and certain neurotransmitters; and is also involved in protein metabolism. Vitamin C is also an important physiological antioxidant and has been shown to regenerate other antioxidants within the body, including alpha-tocopherol (vitamin E). Acute vitamin C deficiency leads to scurvy. The timeline for the development of scurvy varies, depending on vitamin C body stores, but signs can appear within 1 month of little or no vitamin C intake (below 10 mg/day). Initial symptoms can include fatigue (probably the result of impaired carnitine biosynthesis), malaise, and inflammation of the gums.Due to its function as an antioxidant and its role in immune function, vitamin C has been promoted as a means to help prevent and/or treat numerous health conditions.

To emphasize the significance of vitamin C and its various biological activity by consumption of locally available indigenous fruits of Kerala, the present study was carried out to extract vitamin C from fruits like *Phyllanthus emblica* (Gooseberry), *Phyllanthus acidus* (Malay gooseberry), *Averrhoa bilimbi* (Bilimbi), *Syzygium samarangenese* (Rose apple), and vitamin C content of fresh, brined and cooked extracts of these fruits were estimated. The extract rich in vitamin C was screened for phytochemical screening. The major health benefits of vitamin C as anti-lipid peroxidation, anti-inflammatory, anti-diabetic and anti-bacterial were investigated.

Materials and Method

Plant materials:

The fruits from plants such as such as *Phyllanthus emblica* (Gooseberry), *Phyllanthus acidus* (Malay gooseberry), *Averrhoa bilimbi* (Bilimbi) and *Syzygium samarangense* (Rose apple) were collected from local market of Palakkad, Kerala.

Extraction of vitamin C

These fruits were crushed using a mortar and pestle and the juice was filtered with a muslin cloth. The extract obtained was taken for analysis.

Estimation of vitamin C

(a) Preparation of iodine solution.

~5.00 g potassium iodide (KI) and ~0.268 g potassium iodate (KIO_3) were dissolved in 200 mL of distilled water in a 400 mL beaker and 30 mL of 3 M sulfuric acid was added. The solution was then poured into a 500 mL graduated cylinder, and diluted to a final volume of 500 mL with distilled water, and mixed thoroughly and transferred to a 600 mL beaker.

(B) Preparation of vitamin-C standard solution.

0.1 mg of vitamin C was weighed using an analytical balance and diluted to a volume of 100mL in a 250 mL volumetric flask using distilled water.

(C) Standardization of the iodine solution with the vitamin C standard solution.

25.00 mL of vitamin C solution was added into a 125 mL Erlenmeyer flask, to this 10 drops of 1 % starch solution was addded.The burette was rinsed twice with 5 -10 mL of iodine solution and then filled with the same. The initial burette reading was noted. The solution was titrated until the endpoint is reached (the first sign of blue color that remains after at least 20 s of swirling). The final volume was recorded.

(D) Analysis of fresh fruit extract

50.00mL of fresh fruit extract of *Phyllanthus emblica* (Gooseberry), *Phyllanthus acidus* (Malay goosebery), *Averrhoa bilimbi* (Bilimbi) and *Syzygium samarangense* (Rose apple) were pipetted into a 125 mL Erlenmeyer flask , about 10 drops of starch indicator was added to the sample. The solutions were titrated until the endpoint was reached (the first sign of blue color that remains after at least 20 s of swirling). The final volume was recorded.

(E) Analysis of brined fruit extract

The whole fruits were kept in a brined solution for 2-3 days in a dark storage container. 50.00mL of brined fruit extract of *Phyllanthus emblica* (Gooseberry), *Phyllanthus acidus* (Malay goosebery), *Averrhoa bilimbi* (Bilimbi) and *Syzygium samarangense* (Rose apple) were pipetted into a 125 mL Erlenmeyer flask , about 10 drops of starch indicator was added to the sample. The solutions were titrated until the endpoint

was reached (the first sign of blue color that remains after at least 20 s of swirling). The final volume was recorded.

(F) Analysis of cooked fruit extract

The whole fruits were cooked in water and 50.00mL of cooked fruit extract *Phyllanthus emblica* (Gooseberry), *Phyllanthus acidus* (Malay goosebery), *Averrhoa bilimbi* (Bilimbi) and *Syzygium samarangense* (Rose apple) were pipetted into a 125 mL Erlenmeyer flask , about 10 drops of starch indicator was added to the sample. The solutions were titrated until the endpoint was reached (the first sign of blue color that remains after at least 20 s of swirling). The final volume was recorded.

Phytochemical screening of *Phyllanthus emblica* (Gooseberry)

Qualitative Phytochemical Analysis was carried out for the extract of *Phyllanthus emblica* (Gooseberry) as per standard methods described by Brain and Turner (1975) and Evans (1996).

Determination of biological activity

Anti-lipid peroxidative activity TBARS assay

TBARS assay was performed by the method of Ohkowa *et al*., 1979. Egg homogenate (0.5mL of 0.1v/v) and 0.1ml of varying concentration 25, 50.75,100 µg/ml of *Phyllanthus emblica* (Gooseberry) extract were added to a test tube and made up to 1ml with distilled water, 0.5ml of 0.07M ferrous sulphate was added to induce lipid peroxidation and the mixture was incubated for 30 minutes. Then, 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate were added, the resulting mixture was vortexed and then heated at 95^0C for 1 hour. After cooling, 5.0ml of butanol was added to each tube and centrifuged at 3000rpm for 10 minutes. The absorbance of the organic upper layer was measured at 532nm. Inhibition of lipid peroxidation percent by the extract was calculated.

Percentage Inhibition = 100-[(A1/A2) x100]

where A1 is the absorbance value in the presence of extract and A2 of the fully oxidized control.

***Invitro* anti-inflammatory assay**

Inhibition of Protein denaturation assay

Invitro anti-inflammatory activity of gooseberry extract was carried out by the method of Chandra *et al.*, 2012.The reaction mixture 5ml consisted of 0.2ml of egg albumin, 2.8ml of Phosphate buffered saline and 2 ml of varying concentration 25, 50, 75, 100 µg/ml of *Phyllanthus emblica* (Gooseberry) extract. A similar volume of double distilled water served as the control. Next the mixtures were incubated at 37 ± 2°C in a BOD incubator for 15 minutes and then heated at 70°C for 5 minutes. After cooling, their absorbance was measured at 660nm by using the vehicle as blank. Diclofenac sodium was used as the reference drug

and treated similarly for the determination of absorbance. The percentage inhibition of protein denaturation was calculated using the formula

Percentage inhibition = $[1-V_t/V_c] \times 100$

Where V_t is the absorbance of test sample, V_c is the absorbance of control, The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against various concentration of the extract used.

***Invitro* anti-diabetic assay**

Inhibition of Alpha-Amylase Enzyme

The assay was performed by the method of Sheikh, J.H *et al,* 2008. DNS (3, 5 Dinitrosalicylic acid) method was performed to determine the α-Amylase inhibitory activity, by quantifying the reducing sugar (glucose equivalent) liberated under the assay condition. The enzyme inhibitory activity was expressed as decrease in units of glucose liberated (Bhutkar and Bhise, 2012). *Phyllanthus emblica* (Gooseberry) concentrations ranging from 25, 50, 75, 100 µg/ml was incubated with 1ml of crude α-amylase enzyme for 30minnutes at 37°C. After incubation 1ml of 1% buffered starch was added and the mixture was further incubated for 10minutes at room temperature. The reaction was stopped by adding 1ml DNS reagent and the contents were heated in boiling water bath for 5minutes. Blank was prepared without plant extract and enzyme which was replaced with equal quantity of 0.1M phosphate buffer. Control representing 100% enzyme activity without plant extract was also included. The absorbance was read at 540nm using UV Spectrophotometer. Standard antidiabetic drug Acarbose was used as positive control. The antidiabetic property was determined through inhibition of alpha amylase which was expressed as percentage of inhibition and calculated by following equation.

$$\text{Percentage of Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Anti-bacterial activity

Disc diffusion method

In vitro antibacterial screening is generally performed by disc diffusion method (Barry, 1986) for primary selection of the compounds as therapeutic agent. Disc diffusion method is equally suited to screening of antibiotics or the products of plant evaluation (Bauer *et al.*,1996) and is highly effective for rapidly growing microorganisms and the activities of the test compounds are expressed by measuring the diameter of the zone of inhibition. The overnight cultures of *Pseudomonas sp., Aeromonas sp., Escherichia coli, Salmonella sp., Shigella sp.*,were swabbed on the surface of dried MHA agar surface using cotton swabs.

The fruit extracts in varying concentration (10µg/ml, 20µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml) were loaded to the discs and placed on the agar surface and incubated at 37ºC for 24 hours. The diameters of the inhibition zones were measured and compared with that of the standard drug ampicillin.

Results

Estimation of vitamin C

Sl.no	**Fruit extract**	**Volume of iodine used (mL)**	**Volume of extract taken (mL)**	**Concentration of vitamin c (g/L)**	**Amount of vitamin C present in 1L of sample (g)**
Fresh extract					
1	*Phyllanthus emblica* (Gooseberry)	39.5	25	0.0158	2.80
2	*Phyllanthus acidus* (Malay gooseberry)	3.5	15	0.0020	0.41
3	*Averrhoa bilimbi* (Bilimbi)	2.8	25	0.0011	0.20
4	*Syzygium samarangense* (Rose apple)	1.5	10	0.0015	0.26
Brined fruit extract					
5	*Phyllanthus emblica* (Gooseberry)	6.2	8.2	0.0080	1.33
6	*Phyllanthus acidus* (Malay gooseberry)	4.0	25	0.0016	0.28
7	*Averrhoa bilimbi* (Bilimbi)	1.5	15.0	0.0010	0.17

8	*Syzygium samarangense* (Rose apple)	2.6	19.6	0.0010	0.23
Cooked fruit extract					
9	*Phyllanthus emblica* (Gooseberry)	2.1	20	0.0011	0.18
10	*Phyllanthus acidus* (Malay gooseberry)	2.0	25	0.001	0.14
11	*Averrhoa bilimbi* (Bilimbi)	0.7	10	0.0010	0.12
12	*Syzygium samarangense* (Rose apple)	0.8	10	0.001	0.14

Phytochemicals in the extract of *Phyllanthus emblica* (Gooseberry)

Sl.No	**Phytochemicals**	**Aqueous Extract of *Phyllanthus emblica* (Gooseberry)**
1	Alkaloids	+
2	Flavanoids	+
3	Steroids	+
4	Terpenoids	+
6	Phenols	+
7	Saponin	-
8	Tannin	+
9	Carbohydrates	+

10	Proteins and amino acids	+
11	Oils and resins	+

Determination of biological activity

Anti-lipid peroxidative activity TBARS assay

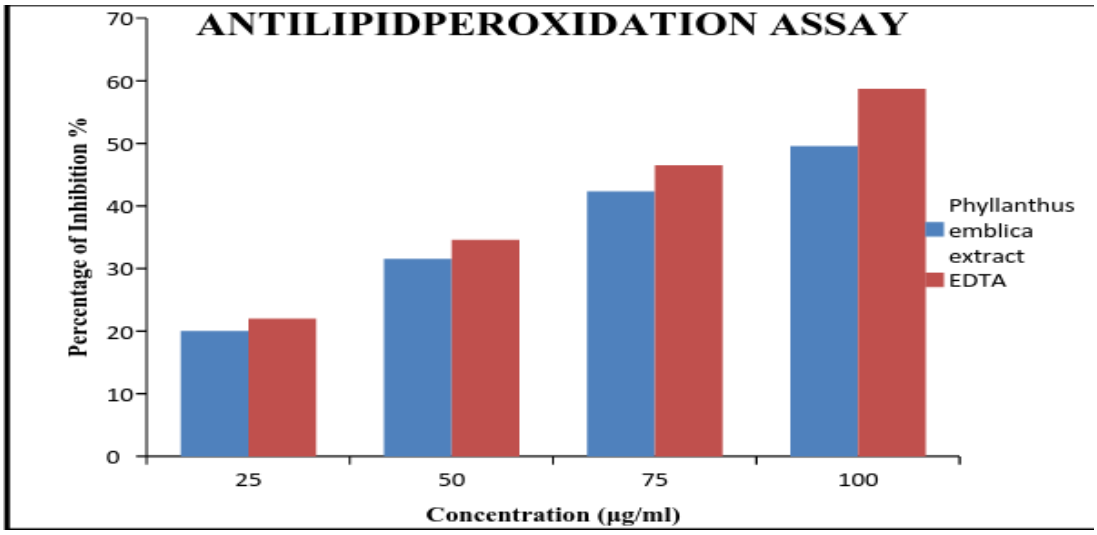

Figure 1 Anti-lipid peroxidation assay of *Phyllanthus emblica* (Gooseberry) extract

***In vitro* anti-inflammatory assay**

Inhibition of protein denaturation assay

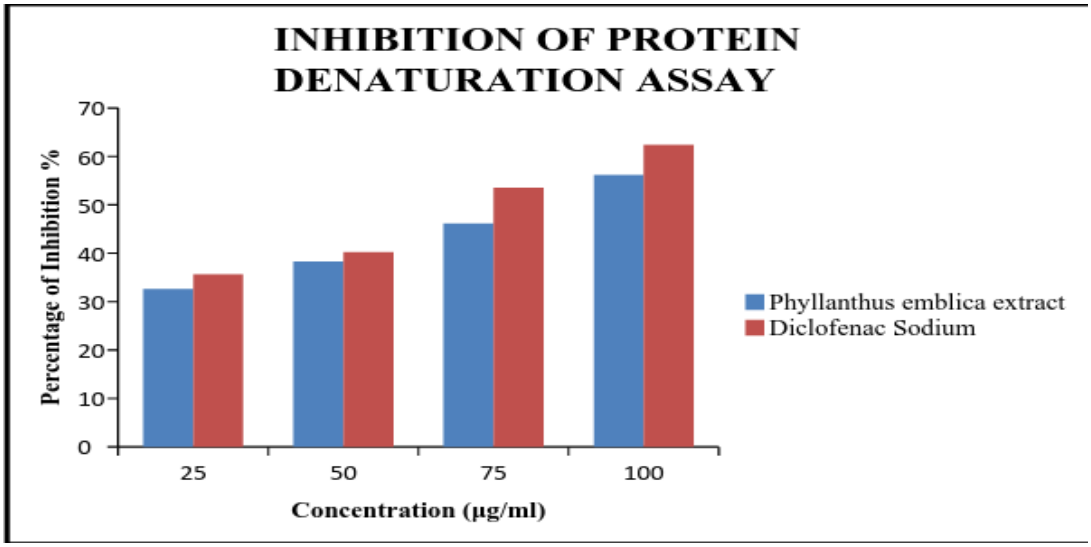

Figure 2 *In vitro* Anti-inflammatory activity of *Phyllanthus emblica* (Gooseberry) extract

***In vitro* anti-diabetic assay**

Inhibition of alpha-amylase enzyme

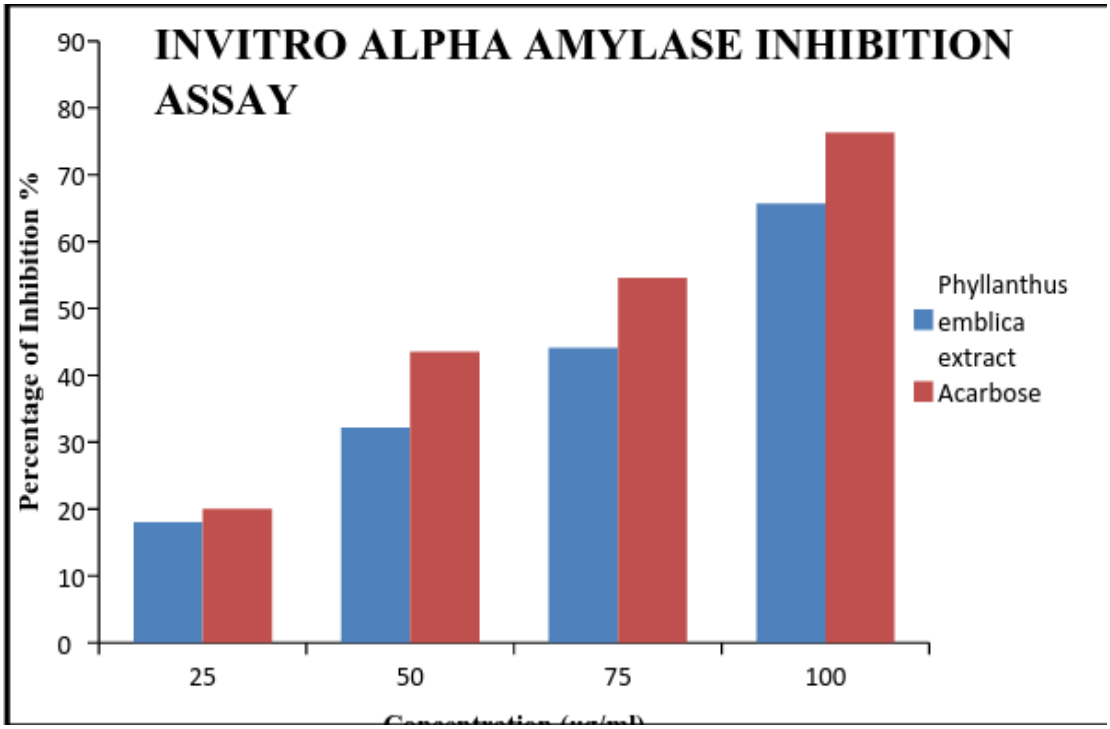

Figure 3 *Invitro* Anti-diabetic activity of *Phyllanthus emblica* (Gooseberry) extract

Antibacterial activity

Disc diffusion assay

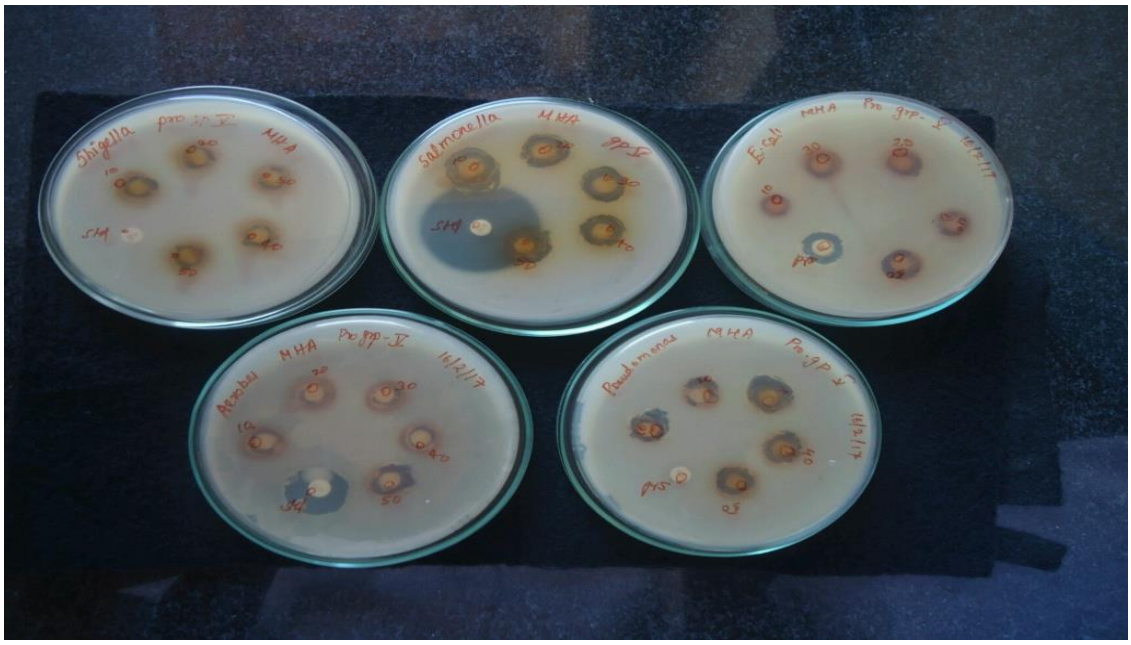

Figure 4 Antibacterial activity *Phyllanthus emblica* (Gooseberry) extract

Microorganisms	Diameter of Zone of inhibition (mm) exerted by different concentration of the extract (µg/ml)					Diameter of Zone of inhibition (mm) exerted by the standard drug Ampicillin (25 mcg)
	10	20	30	40	50	
Escherichia coli	1.0	1.2	1.1	1.1	1.4	1.6
Pseudomonas sp	1.3	1.3	1.6	1.4	1.2	0.7
Aeromonas sp	1.2	1.1	1.2	1.1	1.1	1.8
Shigella sp	1.1	1.0	1.1	1.2	1.1	0.7
Salmonella sp	1.2	1.1	1.1	1.2	1.0	1.9

Table 1 Measurements of zone of inhibition

Discussion:

Estimation of vitamin C

It was observed that the content of vitamin C was found to be higher in the fresh extract of all selected fruits. Among all the fruits, *Phyllanthus emblica* (Gooseberry) showed maximum vitamin C content. The fruits when kept in brine solution for 2-3 days, a remarkable decrease in the vitamin C content were noted. On cooking the fruits, the Vitamin C content gets denatured; as a result there is a great loss of vitamin C. Therefore, for further analysis fresh extract of *Phyllanthus emblica* (Gooseberry) was choosen as it contained maximum amount of vitamin c (~2.8g in 1 litre of extract). Fresh extract of *Phyllanthus emblica* (Gooseberry) was analysed for its phytochemical screening and biological activities.

Phytochemicals in the extract of *Phyllanthus emblica* (Gooseberry)

Phytochemical screening of fresh extract of *Phyllanthus emblica* (Gooseberry) showed the presence of alkaloids, flavonoids, steroids, phenols, terpenoids and tannins whereas saponin was found to be absent in the extract. Phytochemical constituents such as alkaloids, flavonoids, phenols and several other aromatic compounds are secondary metabolites of plants that contribute to its major health benefits.

Determination of biological activity

Anti-lipid peroxidative activity TBARS assay

At a concentration of 100µg/ml, the inhibition of *Phyllanthus emblica* (Gooseberry) extract was 49.56% and that of the standard EDTA was 58.74% . Lipid peroxidation, which is widely recognized as

primary toxicological event, is caused by the generation of free radicals from a variety of sources including organic hydro peroxides, redox cycling compounds and iron-containing compounds. The TBARS assay has been used to measure the degree of lipid peroxidation. TBA reacts specifically with malondialdehyde (MDA), a secondary product of lipid peroxidation to give a red chromogen, which may then be determined spectrophotometrically. This assay revealed that the extracts might prevent reactive radical species from damaging biomolecules such as lipoprotein, DNA, amino acids, sugar, proteins and PUFA in biological and food systems. Vitamin C is an excellent source of electrons therefore it "can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their reactivity" (Bendich, 1990). Vitamin C protects the DNA of the cells from the damage caused by free radicals and mutagens. It prevents harmful genetic alterations with in cells and protects lymphocytes from mutations to the chromosomes (Gaby and Singh, 1991).

***In vitro* anti-inflammatory assay**

Inhibition of protein denaturation assay

The extract of *Phyllanthus emblica* (Gooseberry) exerted an inhibition of 56.18% at a maximum concentration of 100µg/ml, when compared with that of the standard Diclofenac sodium with an inhibition of 62.43% at a maximum concentration 100µg/ml. The denaturation of proteins is a well-documented cause of inflammation and rheumatoid arthritis and hence, this assay was adopted in assessing the properties of the extract in stabilizing the protein from the denaturation process. Several anti-inflammatory drugs have been reported to show dose dependent ability to inhibit thermally induced protein denaturation (Grant *et al.*, 1970).The extract also exhibited a dose dependent ability to inhibit protein denaturation. studies have shown that vitamin C concentration in the blood from rheumatoid arthritis patients are extremely low and that vitamin C may protect against further damage to inflamed joints (Halliwell 1987, Williams *et al.*, 2008).

***In vitro* anti-diabetic assay**

Inhibition of alpha-amylase enzyme

The extract of *Phyllanthus emblica* (Gooseberry) exerted an inhibition of 65.72% at a maximum concentration of 100µg/ml, when compared with that of the standard Acarbose with an inhibition of 76.32% at a maximum concentration 100µg/ml. Alpha-amylase catalyses the hydrolysis of alpha- 1,4-glycosidic linkages of starch, glycogen and various oligosaccharides. Alpha glucosidase further breaks down the disaccharides to simple sugars, readily available for intestinal absorption. The inhibition of their activity in the digestive tract of humans is considered to be effective tool to control diabetes. The extract of *Phyllanthus emblica* effectively inhibited alpha amylase in a dose dependent manner. It has been reported that diabetic individual have low levels of vitamin C in the plasma and in the white blood cells (Cunningham *et al.*, 1991),

which constitute our immune defense. Large-scale clinical trials are needed to determine whether supplementation with large doses of the vitamin is beneficent or not. Some smaller trials have found that supplementation with 2 g/d lowered fasting glucose levels (a beneficial effect) and reduced capillary fragility in diabetics. Mega doses of vitamin C may, however, be toxic in diabetics with certain kidney disorders (Will and Tyers, 1996). It is suspected that vitamin C helps the body to reduce glycolysation, which is an abnormal attachment of sugars to proteins. It also lowers accumulation of the sugar sorbitol (Will and Tyers, 1996), which can damage eyes and kidneys.

Antibacterial activity

Disc diffusion assay

The fruit extracts of *Phyllanthus emblica* (Gooseberry) tested for antibacterial activity against certain bacteria like *Escherichia coli, Pseudomonas sp., Aeromonas sp., Salmonalla sp.and Shigella sp.* produced zone of inhibition. The extracts showed varied inhibitory activity against test organisms. The degree of inhibition was designated as moderate inhibition. The organisms were moderately inhibited by different concentration of the extract. The maximum inhibition zone was created at a concentration of 30µg/ml against *Pseudomonas sp.* with a diameter of 1.6mm. With its antioxidant and immunity-enhancing abilities, vitamin C is an excellent supplement for HIV patients, as it may help with disease resistance and overall well-being (Cathcart, 1984). Vitamin C also increases the acidity of urine, making it in an inhospitable host for bacteria. This may decrease the incidence of urinary tract infection (UTI) (Axelrod, 1985).

Vitamin C is a highly water-soluble compound that has both acidic and strong reducing properties. It naturally occurs in many plants and animals except in humans. Vitamin C being essential constituent for the normal functioning of the body, the study was aimed to analyse the vitamin C content in some of the indigenous fruits of Kerala like *Phyllanthus emblica* (Gooseberry), *Phyllanthus acidus* (Malay gooseberry), *Averrhoa bilimbi* (Bilimbi) and *Syzygium samarangenese* (Rose apple). Vitamin C content was analysed in fresh, brined and cooked fruit extracts. The fresh fruit extract of *Phyllanthus emblica* (Gooseberry) exhibited more Vitamin C content which was selected for the further studies. The fresh extract of Phyllanthus *emblica* (Gooseberry) was screened for its phytochemicals. Major phytochemicals like phenols, flavonoids, alkaloids, tannins and terpenoids were found to be present in the extract.

The fresh extract of *Phyllanthus emblica* (Gooseberry) was studied for certain biological activities like anti-lipid peroxidation, anti-inflammatory, anti-diabetic and anti-bacterial activity. The extract exhibited a dose dependent inhibition of free radicals responsible for lipid peroxidation, thermal denaturation of proteins responsible for inflammation and inhibition of alpha amylase that contribute to diabetes. It also

exhibited moderate inhibition against certain bacteria. The biological activities contributed by the extract in anti-lipid peroxidation, anti-inflammatory, anti-diabetic and anti-bacterial activity may be due to its high Vitamin C content and also due to the presence of secondary metabolites like phenols, flavonoids, tannins, alkaloids and terpenoids.

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