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ORIGINAL ARTICLE

Evaluation of Antiangiogenic Potential of *Psidium guajava* Leaves using *In-Ovo* Chick Chorioallantoic Membrane Assay

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ABSTRACT

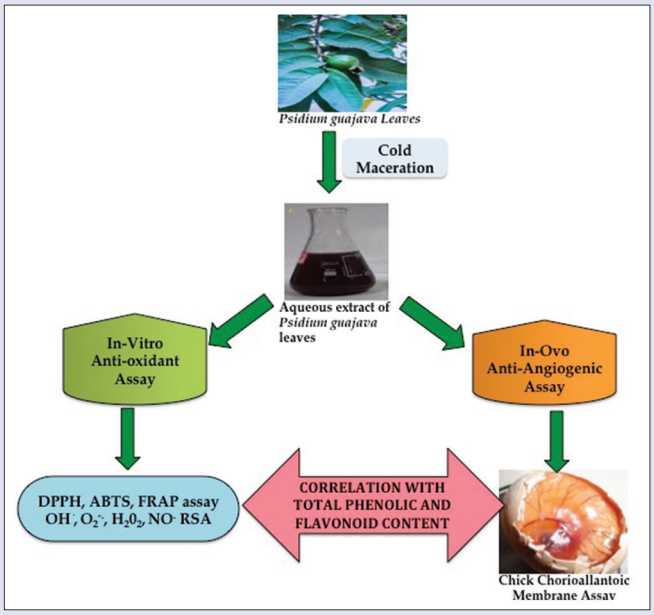
Background: Angiogenesis is the process of formation of new blood vessels from the existing one. Pathological angiogenesis is widely implicated in many diseases, including cancer, diabetic neuropathy, retinopathy, obesity, and arthritis. **Objective:** The present study was aimed to evaluate the *in vitro* antioxidant and *in ovo* antiangiogenic activity of aqueous extract of *Psidium guajava* leaves (AEPG). **Materials and Methods:** *Psidium guajava* commonly known as guava reported to contain polyphenols and flavonoids such as gallic acid, epigallocatechin, catechin, rutin, and quercetin in glycosidic forms in its leaves. The antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), nitric oxide, hydrogen peroxide, hydroxyl, and superoxide radical scavenging assays (RSAs) and antiangiogenic activity was evaluated using vascular endothelial growth factor (VEGF)-induced chick chorioallantoic membrane (CAM).The correlation between the antioxidant and antiangiogenic activity was correlated with total phenolic content (TPC) of AEPG. **Results:** The TPC of AEPG was found to be 493.8 ± 8.9 mg of GAE/g. The total flavonoid content of AEPG was found to be 254.9 ± 13.7 mg of CE/g. *In vitro* antioxidant activity of AEPG showed IC_{50} values of 19.4 ± 1.9, 25.5 ± 0.2, 4.9 ± 0.5, 29.9 ± 2.06, 39.5 ± 2.07, and 29.9 ± 0.9 μg/ml, respectively, for DPPH, ABTS, nitric oxide, hydrogen peroxide, hydroxyl, and superoxide RSAs. Significant reduction in angiogenesis in the AEPG treated groups when compared to untreated VEGF groups and the Pearson's correlation coefficient between TPC of AEPG and total length, area, branches of blood vessels and CAM thickness were −0.9261, −0.9807, −0.9637, and −0.9597, respectively. **Conclusion:** The results revealed potent antiangiogenic activity of AEPG leaves and exhibit significant correlation between the antioxidant and antiangiogenic activity of AEPG and its TPC.

Key words: Antiangiogenic, antioxidant, chorioallantoic membrane, *psidium guajava*, quercetin, vascular endothelial growth factor

SUMMARY

- Role of Anti-oxidant effect of AEPG on its Anti angiogenic activity using *in ovo* chick CAM assay activity was evaluated and showed good correlation with its total phenolic and flavonoid content.

Abbreviations used: EGF: Epidermal growth factor; FGF: Fibroblast growth factor; G-CSF: Granulocyte colony stimulating factor; IL: Interleukin; INF: Interferon; MMP: Matrix metalloproteinases; NOS: Nitric oxide synthase; PAF: Platelet-activating factor; PAI: Plasminogen activator inhibitor; PDGF: Platelet-derived growth factor; PG-E: Prostaglandin E; RSA: Radical scavenging assay; TFC: Total flavonoid content; TPC: Total Phenolic content; TIMP: Tissue inhibitors of metalloproteinases; TNF-α: Tumor necrosis factor alpha; VEGF: Vascular endothelial growth factor.



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INTRODUCTION

Angiogenesis is the process of formation of new blood vessels from the existing one.[1] During physiological conditions, there is a tight balance exist between the angiogenic stimulators (Growth factors such as angiogenin, angiotropin, epidermal growth factor, fibroblast growth factor (FGF), granulocyte colony stimulating factor, platelet-derived growth factor, tumor necrosis factor alpha, vascular endothelial growth factor (VEGF), etc., cytokines such as interleukin-1 (IL-1), IL-6, IL-8, proteases such as matrix metalloproteinases 2 (MMP-2), MMP-9, and positive modulators such as angiopoietin-I, angiostatin II, endothelin,

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erythropoietin, hypoxia, nitric oxide synthase, platelet-activating factor, prostaglandin E, thrombopoietin, etc.) and angiogenic inhibitors (tissue inhibitors of metalloproteinases [TIMP-1], TIMP-2, plasminogen activator inhibitor-I, IL-10, IL-12, angiopoietin-II, endostatin, interferon-α, thrombospondin, etc.). However, in pathological conditions, there may be an imbalance between pro- and anti-angiogenic regulators leading to a marked increase in angiogenesis which is implicated in various diseases including cancer, autoimmune disorders, obesity, psoriasis, diabetic retinopathy, asthma, inflammatory bowel disease, liver cirrhosis, arthritis, and diabetic nephropathy.[2-4]

Targeting angiogenesis would be a plausible therapeutic target for diseases caused by pathological angiogenesis. Recently, US-Food and Drug Administration approved few anti-angiogenic drugs targeting various pathways of angiogenesis such as endothelial growth factor inhibitors, endothelial cell (EC) proliferation inhibitors, EC signaling inhibitors, MMPs inhibitors, EC survival inhibitors, and endothelial precursor cells inhibitors. However, modulating regulatory mechanisms of angiogenesis lead to serious adverse effects such as hypertension, bleeding, thrombocytic events, proteinuria, lymphopenia, leukopenia, and hypothyroidism.[5-7] Hence, the search for plant-based anti-angiogenic therapy becomes essential.

Plant-based drugs have been used in the treatment of many diseases. Plant polyphenols including flavonoids are secondary metabolites present in leaves, fruits, and flowers, possesses anti-oxidant property. Hence, humans consume them for its beneficial effects in the prevention of diseases caused by oxidative stress (OS).

Free radicals (FR) are highly reactive species, generated during the endogenous cellular process by enzymatic and nonenzymatic reactions or by exogenous exposure to radiation, toxins, certain drugs, smokes, and pollutants. However, they are indispensable in host defence mechanism, cell maturation, and cellular intermediate signaling mechanisms. When FR are produced in excess quantities, leads to OS causing deleterious effects to cellular biomolecules including, lipids, proteins, lipoproteins, DNA and is widely implicated in diseases such as cancer, cardiovascular diseases, diabetes, and neurodegenerative diseases. Endogenous antioxidant enzymes superoxide dismutase (SOD), ctalase, glutathione (GSH), and exogenous substances such as Vitamin C, Vitamin E, Omega 3, Omega 6 fatty acids, carotenoids, and flavonoids are utilized in the management of disease caused by OS.[8-11]

Plant-derived polyphenols such as resveratrol, epigallocatechin-3-gallate obtained from red wine, and green tea, respectively, were shown to possess antioxidant and antiangiogenic activity. The anti-angiogenic activities of polyphenols including flavonoids are due to inhibition of the several pathways of the angiogenic process.[4,12-15]

Psidium guajava Linn. (Family– *Myrtaceae*) commonly known as Guava is a tropical tree found in almost every state of India. Conventionally, its leaves, fruits, flowers, barks, and roots are used in diarrhea, dysentery, ulcers, gingivitis, bleeding gums, and toothache due to its astringent and cooling effects. High quantity of polyphenols and flavonoids such as gallic acid, epigallocatechin, catechin, rutin, and quercetin in its glycosidic forms, etc., were reported as the main active constituent of *P. guajava* leaves.[16-18] Hence, this study was planned to evaluate the *in vitro* antioxidant activity and *in ovo* antiangiogenic activity of aqueous extract of *P. guajava* leaves using chorioallantoic membrane (CAM) and correlating with its total phenolic and flavonoid contents.

MATERIALS AND METHODS

Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), (ABTS) ascorbic acid, deoxyribose, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethylene diamine tetra acetic acid (EDTA), Folin–Ciocalteu (FC) reagent (Merck), Gallic acid, nicotinamide adenine dinucleotide (NADH), nitrobluetetrazolium (NBT), N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD), phenazin methosulfate (PMS), Quercetin (Sigma), thiobarbituric acid (TBA), trichloro acetic acid (TCA), 2, 4, 6-tripyridyl-s-triazine (TPTZ), VEGF (Biovision, USA), hematoxylin, eosin, and all other chemicals used were of analytical grade.

Collection and authentication of *Psidium guajava* leaves

The fresh leaves of *P. guajava* were collected from the residential campus area of DIPSAR, New Delhi, India. The leaves were washed with tap water, spread as a layer and dried under shade at room temperature. The crude leaves were authenticated by Dr. Sunita Garg, Chief scientist, Raw Material Herbarium and Museum, Delhi (RHMD), Council of Scientific and Industrial Research, National Institute of Science Communication and Information Resources (NISCAIR), PUSA Campus, New Delhi vide Reference Number NISCAIR/RHMD/Consult/2014/2547/126-2 Dated 24/11/2014.

Preparation of aqueous extract of *Psidium guajava* leaves

The shade dried and coarsely powdered *Psidium guajava* leaves (1000 g) were extracted by cold maceration method with distilled water. The mixture was shaken for 24 h using a mechanical shaker. After maceration, the extract was filtered using muslin cloth, and the filtrate, aqueous extract of *Psidium guajava* (AEPG) was lyophilized and stored in a vacuum desiccator for further use.

Determination of total phenolic content

The total phenolic content (TPC) in the AEPG was determined by FC method,[19,20] using gallic acid as a reference standard. Briefly to 200 µl of different concentration of gallic acid (20–200 µg/ml) and 500 µg/ml of AEPG, 1 ml of 0.2 mol/L FC reagent was added and mixed well. After 4 min, 800 µl of 1 M $Na_2CO_3$was added and incubated at room temperature for 1 h. The absorbance was read at 765 nm using ultraviolet-visible (UV/VIS)-spectrophotometer and TPC of the AEPG was calculated from the standard curve and expressed as mg of gallic acid equivalence (GAE)/g of extract.

Determination of total flavonoid content

The total flavonoid content (TFC) of AEPG was estimated using aluminum chloride complex (λ_{max} at 510 nm) formation in the presence of $NaNO_2$ in alkaline medium[21] using catechin as a reference standard. Briefly to 1 ml of different concentration of catechin (20–200 µg/ml) and 500 µg/ml of AEPG, 4 ml of water and 300 µl of 5% $NaNO_2$ were added in a 10 ml volumetric flask. After 5 min, 300 µl of 10% $AlCl_3$ was added and mixed well. After 6 min, 2 ml of 1 M NaOH was added, and the volume was made up to 10 ml with deionized water, and the absorbance was read at 510 nm using UV/VIS-spectrophotometer against blank. TFC of the AEPG was calculated from the standard curve and expressed as mg of catechin equivalent (CE)/g of extract.

In vitro antioxidant assay

Determination of 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity

DPPH is a stable FR with an odd electron that shows paramagnetism and appears deep violet color in ethanol (λ_{max} 517 nm).[22] The DPPH radical scavenging activity of AEPG, quercetin and ascorbic acid was estimated according to the previously reported method.[22,23] Briefly,

0.1 mM DPPH in ethanol was prepared and different concentrations (5-200 µg/ml, namely, 5, 10, 20, 40, 80, 120, 160, and 200 µg/ml) of AEPG, quercetin and ascorbic acid were prepared. To 1 ml of varying concentrations of AEPG, quercetin or ascorbic acid in methanol, 3 ml of 0.1 mM DPPH solution was added. After 30 min, the absorbance was read spectrophotometrically at 517 nm. Ascorbic acid was used as the reference standard 0.1 mM DPPH in ethanol served as blank. DPPH radicals scavenging activity of the test solution was expressed as the percentage inhibition of FR.

Determination of ferric reducing antioxidant potential value

Reduction of Ferric (Fe-III)–TPTZ to Ferrous (Fe-II)-TPTZ at acidic pH forms blue color (λ_{max} 593 nm). The FRAP assay of AEPG was performed as per previously reported method with little modifications.[19,24] FRAP reagent was prepared freshly by mixing 300 mM acetate buffer, 10 mM TPTZ, 20 mM Ferric chloride in the ratio of 10:1:1 (100, 10, 10 ml) and warmed to 37° C for 10 min before use and served as blank. 1 mM Ferrous sulfate stock solution was diluted to prepare five concentrations to plot standard curve. Briefly, 100 µl of the sample and different concentration of standards were diluted with 300 µl of water and mixed well, followed by the addition of 3 ml of FRAP reagent and incubated for 4 min. The absorbance was measured at 593 nm using UV/Vis spectrophotometer. The FRAP value of AEPG was calculated from the standard curve prepared from the absorbance of the reaction mixture and known concentration of ferrous sulfate and expressed in term of µM of Fe^{2+}/g of the sample.

Determination of 2,2′-azino-bis (3-ethylbenzo thiazoline-6-sulphonic acid radical scavenging activity

Potassium persulfate oxidizes the ABTS to generate ABTS radical monocation and shows blue/green color (λ max at 734 nm).This assay was performed as per previously reported method.[19,25] Briefly, 1 ml of each solution of 7 mM ABTS and 2.4 mM Potassium persulfate were mixed and stored at room temperature for 12 h in dark. One ml of the freshly prepared above mixture was diluted with 60 ml of methanol, also served as blank. A volume of 100 µl of each of different concentrations (5–200 µg/ml) of AEPG, quercetin or ascorbic acid was made up to 3 ml with the diluted mixture and mixed well. After 7 min, the absorbance was measured at 734 nm using UV/Vis spectrophotometer.

Determination of nitric oxide radical scavenging capacity

This assay was performed using Griess reagent, as per previously reported method.[26,27] Griess reagent A (2% w/v sulphanilamide in 4% Phosphoric acid) Griess Reagent B (0.2% w/v N-(1-napthyl) ethylenediamine dihydrochloride) (NEDD), 10 mM sodium nitroprusside in 20 mM Phosphate Buffer pH 7.4 were prepared. The reaction mixture was prepared by mixing 2 ml of 10 mM sodium nitroprusside, 0.5 ml of phosphate buffer and 0.5 ml of each of different concentrations (5–200 µg/ml) of AEPG, quercetin or ascorbic acid, incubated for 150 min at 25°C. Briefly to the 0.5 ml of the reaction mixture, 0.5 ml of Griess reagent A was added, followed by the addition of 0.5 ml of Griess reagent B, after 5 min. Further, the mixture was incubated at 25°C for 30 min, the absorbance was measured at 542 nm using UV/Vis spectrophotometer. The blank reaction mixture was prepared without the addition of sample or standard; remaining same procedure was used as a test.

Determination of hydrogen peroxide scavenging capacity

This assay was performed according to previously reported methods.[28,29] Briefly, to 1 ml of the 40 mM hydrogen peroxide in PBS solution, 2 ml of different concentrations (5–200 µg/ml) of AEPG, quercetin or ascorbic acid were added. After 10 min, the absorbance of the test samples were recorded at 230 nm using PBS and different concentrations of samples without hydrogen peroxide as blank.

Determination of hydroxyl radical scavenging capacity

This assay was performed according to the previously described method.[30,31] The reaction mixture was prepared by adding the reagents in the following order, 100 µl of 1 mM EDTA, 10 µl of 10 mM $FeCl_3$, 100 µl of 10 mM H_20_2, 360 µl of 10 mM deoxyribose, 1 ml different concentrations (5–200 µg/ml) of AEPG or quercetin, 330 µl of 50 Mm phosphate buffer, and 100 µl of 1 mM ascorbic acid. After incubating the reaction mixture for 1 h at 37° C, it was mixed with 0.5 ml of each of 5% TCA and 1% TBA in 0.025 mM NaOH and placed in a boiling water bath for 30 min. The Pink color thus developed was measured using UV-Vis spectrophotometer at 532 nm against the blank

Determination of Superoxide radical scavenging capacity

PMS– NADH system generates superoxide radical, which when reacted with NBT forms, NBT diformazan, the color intensity is measured at 560 nm. This assay was performed according to the previously reported method.[32] Briefly, 1 ml of each of 16 mM Tris-HCl buffer, 78 µM NADH in tris buffer, and 50 µM NBT were added followed by the addition of 1 ml of each of different concentrations (5-200 µg/ml) of AEPG or quercetin. The reaction was initiated by adding 1 ml of 10 µM PMS. After incubating for 5 min at 25°C, the absorbance was measured using UV-Vis spectrophotometer at 560 nm against the blank.

Calculation of percentage Inhibition and IC_{50} values

DPPH, ABTS, nitric oxide, hydroxyl, superoxide radicals, and H_2O_2 scavenging activity of AEPG, quercetin, and ascorbic acid expressed as percentage inhibition and it was calculated using the following formula:

$$\% \text{ Inhibition} = \left\{\frac{(\text{Absorbance of the control} - \text{Absorbance of the test solution})}{\text{Absorbance of the control}}\right\} \times 100$$

All the experiments were performed in triplicate. The graph was obtained by plotting the varying concentration (5–200 µg/ml) of samples against % Inhibition using graph pad prism. The inhibitory concentration (IC_{50}) is defined as the concentration of sample under test required to inhibit the 50% of the reaction. The log dose-response curve graph was obtained by plotting the log concentration against % inhibition. The equation of straight line ($y = mx + c$) of linear regression analysis was obtained. Then, the IC_{50} value was calculated.

In Ovo antiangiogenic assay using Chicken chorioallantoic membrane

Anti-angiogenic activity of AEPG and quercetin were evaluated using the CAM assay as described earlier[33-35] with slight modifications. Briefly, fresh fertile white leghorn chicken eggs were procured (sanjeev poultry breeding farm, Gurgaon, India) on day 1. Eggs were wiped with 70% ethanol, numbered and incubated at 37°C in a humidified atmosphere. On day 3, a small hole was made in the narrow end of the egg, and 3–5 ml of albumin was withdrawn using sterile disposable syringe. The hole was closed with sterile plaster and eggs were kept inside the incubator. On day 9, a 1 cm^2 window was opened [Figure 1a]. Sterile gelatin sponge (Gel sponge-Pfizer) of 1–3 mm was impregnated with PBS 10 µl/sponge in the normal group, VEGF10 ng/10 µl/sponge in the control eggs, and different concentrations of AEPG withVEGF10 ng, i.e., VEGF10 ng/5 µl + AEPG 50 µg/5 µl, VEGF 10 ng/5 µl + AEPG 125 µg/5 µl, VEGF 10 ng/5 µl + AEPG 250 µg/5 µl, VEGF 10 ng/5 µl + AEPG 500 µg/5 µl, and two concentrations of quercetin i.e., VEGF 10 ng/5 µl + QTN 20 µM/5 µl and VEGF 10 ng/5 µl + QTN 40 µM/5 µl. Treated sponges were kept on the CAM through window

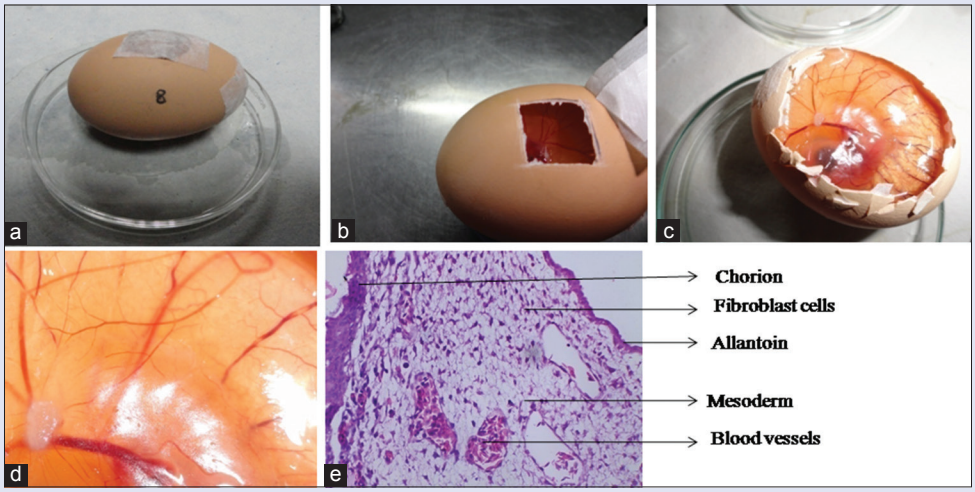

Figure 1: Angiogenic activity evaluation method using chorioallantoic membrane: (a) Fertile White Leghorn chicken egg, numbered (on day 1), Albumin withdrawn through an hole from narrow end of egg (on day 3), 1 Cm^2 window opened (on day 9). (b) 1 cm^2 window opened and Sterile Gelatin sponge placed on the chorioallantoic membrane (on day 9). (c) Wide opened window (on day 12) (d) Photograph of the chorioallantoic membrane showing sponge (e) H and E staining of the chorioallantoic membrane

carefully after confirming the presence of blood vessels [Figure 1b]. The window was covered with micropore plaster and kept inside the incubator for 3 days (72 h). All the procedures were done in strict aseptic condition. On day 12, the window was widened by removing shell [Figure 1c] and checked for the viability of the fetus and photograph was taken [Figure 1d]. These photographs were analyzed for angiogenic/antiangiogenic effect by measuring the total length, size, and branches of blood vessels using "AngioQuant" MATLAB toolbox software (version 6.5, MathWorks, USA). Then, the sponge was fixed by the addition of 10% neutral buffered formalin (NBF) over the sponge and kept aside for few hours. Then, CAM was separated and stored it in 10% NBF, for histopathological evaluation. CAM was dehydrated with ethanol, washed with xylene, and embedded in paraffin wax. Vertical section of 5–6 μm thickness of CAM was taken using microtome and stained with hematoxylin and eosin. The slides were inspected under a microscope (Motic) for changes in the vascular density and photo documentation [Figure 1e]. The thickness of CAM was measured in six locations of H and E-stained CAM each group using MOTIC Images plus 2.0 ML software. The concentration of VEGF 10 ng was used as pro-angiogenic stimuli after validating the concentrations of VEGF, by performing the CAM assay with PBS, PBS + VEGF 2.5 ng, PBS + VEGF 5 ng and PBS + VEGF 10 ng in 10 μl/sponge.

Correlation analysis

Correlation analysis was done to evaluate the relationship between the amount of total phenols (gallic acid equivalent in μg/ml), total flavonoids (catechin equivalent in μg/ml) present in the AEPG (5–200 μg/ml) of samples against FR scavenging assays (% inhibition) using Pearson's correlation coefficient (r value) using graph pad prism. The correlation analysis was also done to evaluate the relationship between the FR scavenging assays used to study the *in vitro* antioxidant activity of AEPG and *r* values are tabulated. The correlation between the anti-angiogenic activity of AEPG and TPC present in the AEPG was also determined by calculating *r* value.

Statistical analysis

All *in vitro* FR scavenging assays were performed in triplicate. The results obtained were expressed as mean ± standard deviation one-way analysis of variance and *t* tests were used. The correlation analysis was performed by calculating the Pearson's correlation coefficient (*r* value).[36] In CAM assay, results were analyzed using ANOVA followed by *post hoc* test of Turkey's multiple comparison tests.[34] The statistical analysis was performed using GraphPad Prism (version 5) software, CA, USA. The results were considered statistically significant if value of $P \leq 0.05$.

RESULTS

Total phenolic contents and total flavonoid content

The TPC in the AEPG was found to be 493.8 ± 8.9 mg of the gallic acid equivalent per gram of the extract. The study showed that the TFC of the AEPG was found to be 254.9 ± 13.7 mg of catechin equivalent per gram of the extract.

Free radical scavenging activity of aqueous extract of *Psidium guajava*, quercetin and ascorbic acid

The inhibition of DPPH FR by AEPG, quercetin, and ascorbic acid, at 5 μg/ml concentration showed 19.43 ± 2.03, 34.78 ± 1.22, and 31.45 ± 0.23% inhibition, respectively, and at 200 μg/ml concentration 94.72 ± 0.46, 93.81 ± 0.68, and 89.56 ± 0.74% inhibition, respectively [Figure 2a]. The IC_{50} value of AEPG, quercetin, and ascorbic acid in DPPH radical scavenging assay (RSA) was found to be 19.4 ± 1.9, 7.7 ± 0.2, and 19.2 ± 0.2 μg/ml, respectively.

The IC_{50} value of AEPG, quercetin, and ascorbic acid in ABTS RSA was found to be 25.5 ± 0.2, 10.52 ± 0.3, and 13.4 ± 1.2 μg/ml, respectively. At 5 μg/ml concentration, AEPG, quercetin and ascorbic acid showed 24.59 ± 1.12, 34.83 ± 1.14, and 36.94 ± 2.27% inhibition, respectively. At 200 μg/ml concentrations, AEPG, quercetin, and ascorbic acid exhibited 86.83 ± 0.14, 90.44 ± 0.65, and 92.27 ± 0.73% inhibition of ABTS radical scavenging, respectively [Figure 2b]. The FRA*P* value of the AEPG was found to be 2655.9 ± 234.9 μM (Fe (II)/g dry mass.

The IC_{50} value of AEPG, quercetin, and ascorbic acid in nitric oxide RSA was found to be 4.9 ± 0.5, 37.07 ± 6.05, and 42.8 ± 9.3 μg/ml, respectively. At 5 μg/ml concentration, AEPG, quercetin and ascorbic acid showed 50.03 ± 0.69, 26.72 ± 2.92, and 34.45 ± 2.61% inhibition, respectively. At 200 μg/ml concentration, AEPG, quercetin and ascorbic acid displayed 75.01 ± 0.99, 73.52 ± 1.06, and 71.51 ± 1.14% inhibition, respectively [Figure 2c].

The IC_{50} value of AEPG, quercetin and ascorbic acid in H_2O_2 scavenging capacity was found to be 29.9 ± 2.06, 12.5 ± 1.7, and 26.7 ± 4.3 μg/ml,

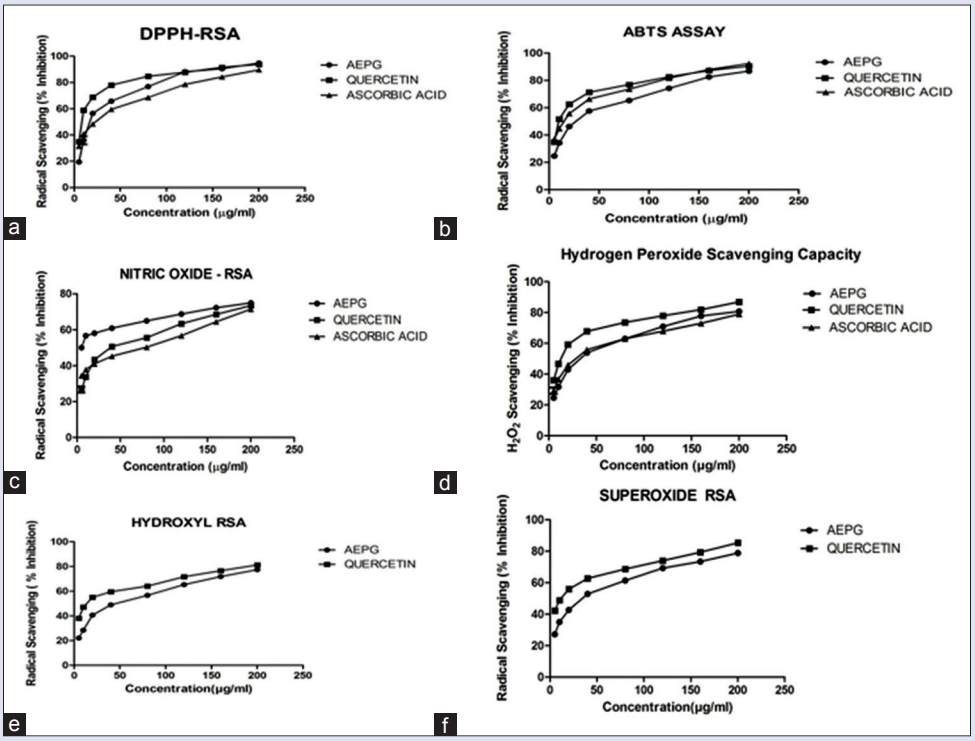

Figure 2: Radical scavenging activity of aqueous extract of *Psidium guajava*, quercetin and ascorbic acid (a) 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (b) 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid radical scavenging assay (c) Nitric Oxide radical scavenging assay (d) H_2O_2 scavenging capacity (e) Hydroxyl radical scavenging assay (f) Superoxide radical scavenging assay

respectively. At 5 µg/ml concentration, AEPG, quercetin and ascorbic acid showed 24.73 ± 1.14, 36.01 ± 2.16, and 29.31 ± 3.45% inhibition. At 200 µg/ml concentrations, AEPG, quercetin and ascorbic acid exhibited 80.79 ± 0.87, 86.93 ± 0.91, and 78.93 ± 0.83% inhibition of H_2O_2 scavenging, respectively [Figure 2d].

The IC_{50} value of AEPG and quercetin in hydroxyl RSA was found to be 39.5 ± 2.07 and 14.2 ± 0.8 µg/ml, respectively. At 5 µg/ml concentration, AEPG and quercetin showed 22.02 ± 1.04 and 38.06 ± 0.17% inhibition, respectively. At 200 µg/ml concentrations, AEPG and quercetin 77.42 ± 2.56 and 81.20 ± 3.29% inhibition of hydroxyl radical scavenging, respectively [Figure 2e].

The IC_{50} value of AEPG and quercetin in superoxide RSA was found to be 29.9 ± 0.9 and 11.4 ± 0.3 µg/ml, respectively. At 5 µg/ml concentration, AEPG and quercetin exhibited 27.24 ± 2.54 and 42.18 ± 0.50% inhibition, respectively. At 200 µg/ml concentrations, AEPG and quercetin showed 78.85 ± 1.99 and 85.38 ± 0.78% inhibition of superoxide radical scavenging, respectively [Figure 2f].

Correlation analysis of total phenolic and flavonoid content with radical scavenging assay

The Pearson's correlation coefficient (*r* value) between the TPC and total flavonoids content present in the AEPG (5-200 µg/ml) samples was 1.000 [Figures 3a and 4a]. The *r*-values between TPC versus DPPH RSA [Figure 3b], TPC versus ABTS RSA [Figure 3c], TPC versus nitric oxide RSA [Figure 3d], TPC versus H_2O_2 scavenging activity [Figure 3e], TPC versus hydroxyl RSA [Figure 3f], and TPC versus superoxide RSA were 0.9792, 0.9830, 0.9966, 0.9748, 0.9920, and 0.9790, respectively.

The *r* values between the TFC and various FR scavenging assays, DPPH RSA [Figure 4b], ABTS RSA [Figure 4c], NO RSA [Figure 4d], H_2O_2 scavenging activity [Figure 4e], hydroxyl RSA [Figure 4f], and superoxide RSA were 0.9792, 0.9830, 0.9966, 0.9748, 0.9920, and 0.9790, respectively. The correlations between TPC, TFC, and DPPH, ABTS, superoxide radical, and H_2O_2 scavenging assays were statistically significant (**$P < 0.01$). Statistically significant (***$P < 0.001$) correlations were observed between TPC, TFC and NO, hydroxyl RSAs.

The *r* value between the DPPH, ABTS, NO, hydroxyl, superoxide radical, and H_2O_2 scavenging assays used to study the *in vitro* antioxidant activity of AEPG were calculated and tabulated in Table 1. The correlations between the *in vitro* antioxidant assays of AEPG were statistically significant (***$P < 0.001$).

Validation of the concentration of vascular endothelial growth factor

The total length, area and branches of the blood vessels of PBS, VEGF 2.5 ng, VEGF 5 ng, and VEGF 10 ng group's CAM photos were quantified in pixels and statistically evaluated [Figure 5a]. There was a significant (**$P < 0.01$) difference in the total length, size, and branches of blood vessels between the PBS and VEGF 10 ng groups; hence VEGF 10 ng was selected for the further studies.

Angio-Quantification of VEGF + AEPG and VEGF + Quercetin Treated chorioallantoic membrane

The total length, area and branches of the blood vessels in the PBS, VEGF 10 ng, V10 + AEPG 50 µg, V10 + AEPG 125 µg, V10 + AEPG 250 µg, V10 + AEPG 500 µg, V10 + Quercetin 20 µM, and V10 + Quercetin 40 µM groups were quantified in pixels and statistically evaluated [Figure 5b]. There was a significant (*$P < 0.05$) decrease in the total length, area, and branches of blood vessels in V10 + AEPG 125 µg and V10 + Quercetin 20 µM groups compared to VEGF 10 ng

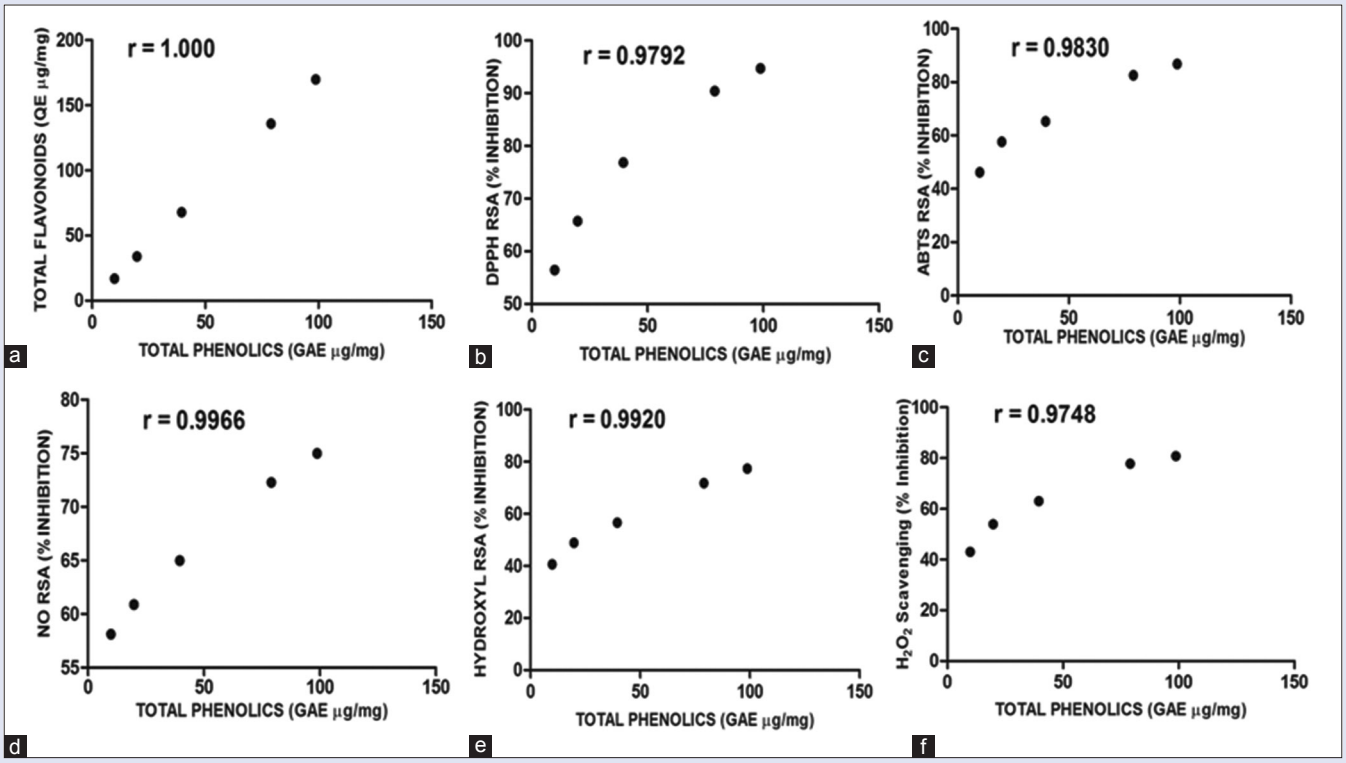

Figure 3: Pearson's correlation analysis between total phenolic content of aqueous extract of *Psidium guajava* (20, 40, 80, 160, 200 µg/ml) and radical scavenging assays. (a) total phenolic content versus total flavonoid content*** (b) total phenolic content versus 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay** (c) total phenolic content versus 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid radical scavenging assay** (d) total phenolic content vs Nitric Oxide radical scavenging assay *** (e) total phenolic content versus hydroxyl radical scavenging assay*** (f) total phenolic content versus H_2O_2 scavenging capacity** (*r* = Pearson's Correlation Coefficient) (***$P < 0.001$, **$P < 0.01$)

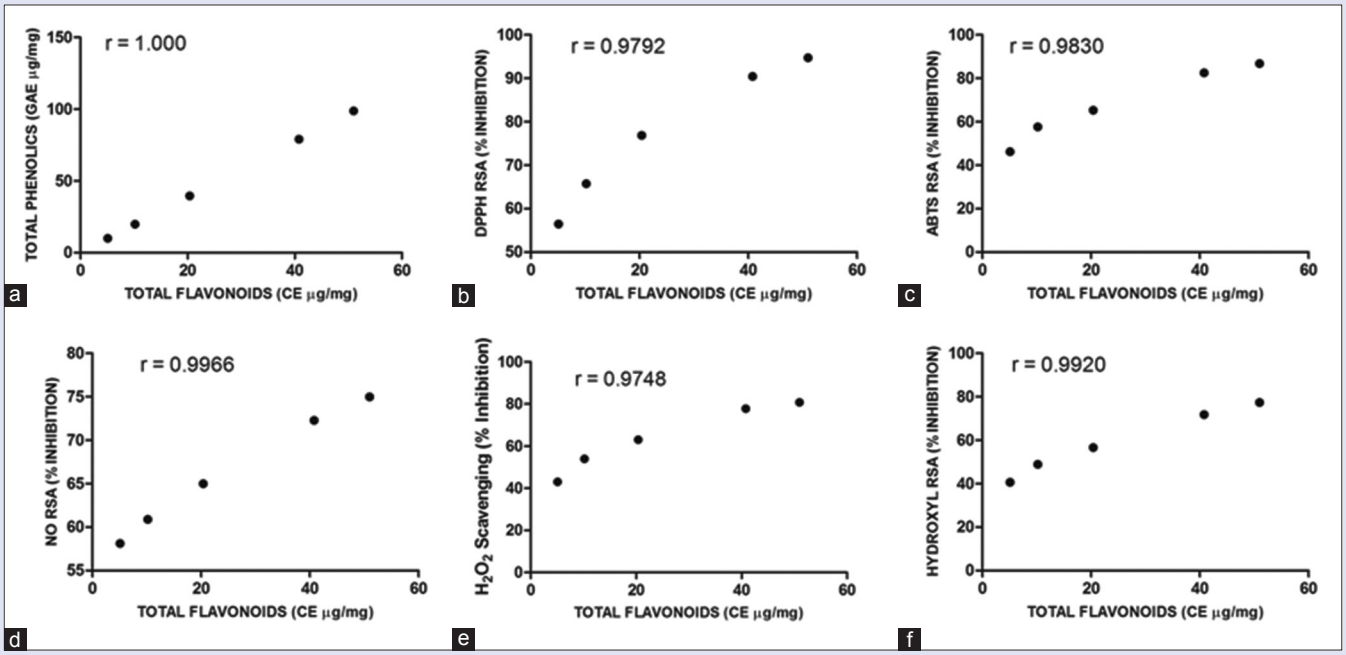

Figure 4: Pearson's correlation analysis between total flavonoid content of aqueous extract of *Psidium guajava* (20, 40, 80, 160, 200 µg/ml) and radical scavenging assays. (a) Total flavonoid content versus total phenolic content*** (b) total flavonoid content versus 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay** (c) Total flavonoid content versus 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid radical scavenging assay** (d) Total flavonoid content versus nitric oxide radical scavenging assay*** (e) Total flavonoid content versus H_2O_2 scavenging capacity** (f) total flavonoid content versus hydroxyl radical scavenging assay*** (r = Pearson's Correlation Coefficient) (***$P < 0.001$, **$P < 0.01$)

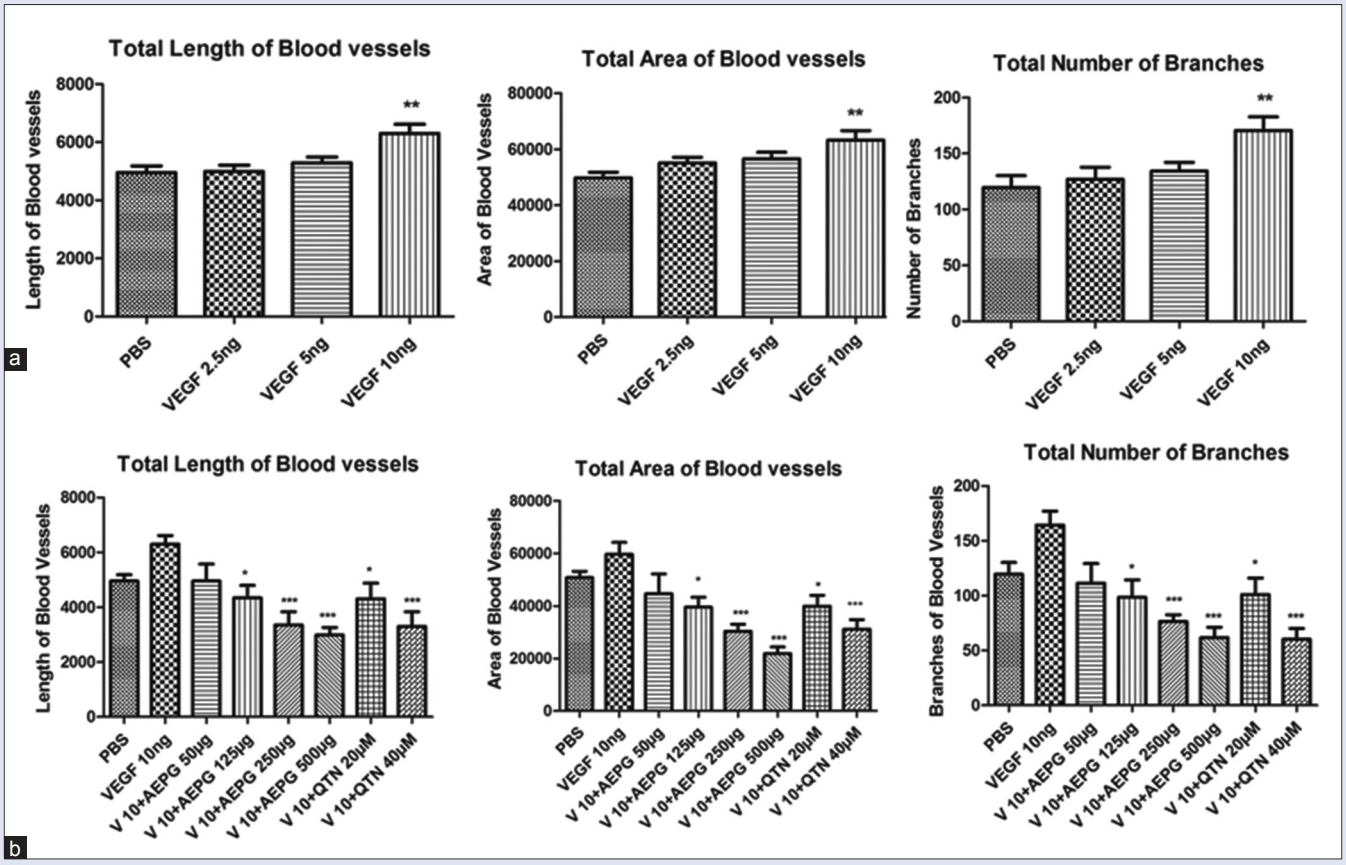

Figure 5: Quantification of Angiogenesis using Angioquant software (a) Validation of the concentration of vascular endothelial growth factor to induce angiogenesis. (b) Evaluation of anti-angiogenic activity of aqueous extract of *Psidium guajava* (50, 125, 250, and 500 µg/ml) and Quercetin (20 µM and 40 µM) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Table 1: Pearson's correlation coefficient (*r*) values between the Radical Scavenging assays of AEPG

	DPPH	ABTS	NO RSA	OH RSA	SO RSA	H_2O_2 SC
DPPH	1.0000	0.9879	0.9665	0.9845	0.9844	0.9872
ABTS	0.9879	1.0000	0.9871	0.9988	0.9979	0.9989
NO RSA	0.9665	0.9871	1.0000	0.9865	0.9880	0.9830
OH RSA	0.9845	0.9988	0.9865	1.0000	0.9976	0.9982
SO RSA	0.9844	0.9979	0.9880	0.9976	1.0000	0.9985
H2O2 SC	0.9872	0.9989	0.9830	0.9982	0.9985	1.0000

(Concentration of AEPG used 5, 10, 20, 40, 80, 120, 160, 200 µg/ml). All the r values are highly significant ($P<0.001$)

group. There was a highly significant (***$P < 0.001$) decrease in the total length, area, and branches of blood vessels in V10 + AEPG 250 µg, V10 + AEPG 500 µg, and V10 + Quercetin 40 µM groups compared to VEGF 10 ng group.

Morphometric analysis of H and E stained CAM

The H and E stained CAMs showed chorion epithelium and endodermic allantoic epithelium with mesodermal fibroblast cells along with the blood vessels [Figures 1e and 6a-h]. There was a significant decrease (*$P < 0.05$) in the CAM thickness of V10 + AEPG 125 µg and V10 + Quercetin 20 µM groups and highly significant decrease (***$P < 0.001$) was noted in the V10 + AEPG 250 µg, V10 + AEPG 500 µg, and V10 + Quercetin 40 µM groups when compared to the thickness of VEGF 10 ng CAMs [Figure 6i].

Correlation analysis of the antiangiogenic activity of aqueous extract of *Psidium guajava* and its total phenolic content

The total length, area, branches of blood vessels, and CAM thickness in V10 + AEPG 50 µg, V10 + AEPG 125 µg, V10 + AEPG 250 µg and V10 + AEPG 500 µg treated groups were correlated with TPC of AEPG. The correlation between TPC versus total length (r =-0.9261) [Figure 7a], TPC versus branches of blood vessels ($r = -0.9637$) [Figure 7c] and TPC versus CAM thickness ($r = -0.9597$) [Figure 7d], showed statistically significant negative correlation (*$P < 0.05$) with its TPC and total area of the blood vessels ($r = -0.9807$) [Figure 7b], showed statistically significant negative correlation (**$P < 0.01$) with its TPC, proving the concentration dependent inhibition of angiogenesis by AEPG.

DISCUSSION

The imbalance between the formation and scavenging of FRs ultimately results in OS, which is a major component in disease states such as diabetes, cancer, cardiovascular disease, neurodegenerative diseases, and aging. Natural plant-based antioxidants are supplemented as nutraceuticals for the prevention of diseases. Plants with significantly higher polyphenolic contents are considered as stronger antioxidants.[37-39]

The TPC of different varieties of leaves of *P. guajava* was reported by Chen and Yen, as Shi Ji Ba 458 ± 8.1, Shui Jing Ba 414 ± 8.2, Tu Ba 483 ± 7.1, Hong Ba 455 ± 6.1 mg of GAE per g.[40] Recent study reported the total

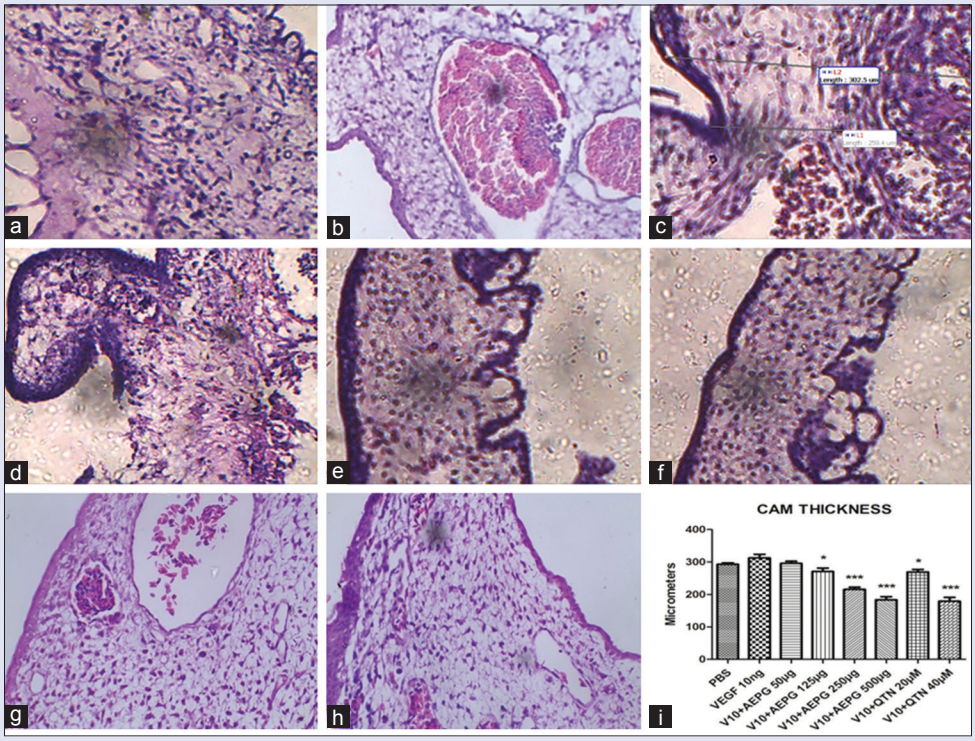

Figure 6: Micro photo analysis of H and E stained chorioallantoic membrane s at ×40.(a) PBS treated chorioallantoic membrane (b) vascular endothelial growth factor 10 ng treated chorioallantoic membrane (c) V10 + aqueous extract of *Psidium guajava* 50 µg treated chorioallantoic membrane (d) V10 + aqueous extract of *Psidium guajava* 125 µg treated chorioallantoic membrane (e) V10 + aqueous extract of *Psidium guajava* 250 µg treated chorioallantoic membrane (f) V10 + aqueous extract of *Psidium guajava* 500 µg treated chorioallantoic membrane (g) V10 + Quercetin20 µM treated chorioallantoic membrane (h) V10 + Quercetin40 µM treated chorioallantoic membrane (i) chorioallantoic membrane Thickness measurement

polyphenolic content of AEPG as 470.0 ± 48.8 mg of GAE/g.[18] The study reveals the presence of significant amount of TPC s, i.e., 493.8 ± 8.9 mg of GAE/g of AEPG. The TFC of AEPG was 254.9 ± 13.7 mg of CE/g of AEPG, which was closer to the amount already, reported, i.e., 248.6 ± 34.2 mg of CE/g.[18] The study revealed the strong positive correlation between TPC versus TFC, *in vitro* antioxidant and RSAs. A study reported the strong correlation between the TPC and DPPH (r = 0.939), ABTS (r = 0.966), and FRAP assays (r = 0.906).[19]

The DPPH assay is the most commonly used and convenient method to evaluate the antioxidant potential of the natural antioxidants.[22] The study displayed that AEPG and quercetin showed almost similar DPPH inhibition in 120, 160, and 200 µg/ml; however, the IC_{50} values of AEPG and ascorbic acid were very close, displaying the antioxidant potential of AEPG. There was a significant correlation between the TPC, TFC, and DPPH assay in the present study. A recent study reported that the DPPH assay strongly correlates with the TPC and ABTS assay with the r = 0.939 and 0.906, respectively.[19]

ABTS assay is one of the most commonly used antioxidant assays in the name of TEAC-Trolox equivalent antioxidant capacity assay. Antioxidant capacity of the test compound is measured as compared to standard Trolox (aqueous soluble vitamin E analog) solution. Our present study displayed a strong correlation between the ABTS assay and TPC, TFC, DPPH, nitric oxide, hydroxyl superoxide, and H_2O_2 scavenging activity. Our results were in agreement with earlier studies which reported strong correlation between ABTS and TPC (r = 0.97) FRAP assay (r = 0.97), and DPPH assay (r = 0.85) in guava fruit extracts.[36]

The FRAP assay is used, for evaluation of *in vitro* antioxidant activity, as it was simple, highly reproducible, rapidly performable assay and showed high correlation (r = 0.97) with total phenolics while determining the antioxidant activity in *P. guajava* fruit extract.[36] Another study also showed the strong correlation between FRAP and ABTS assay (r = 0.946).[19] The findings from our current study showed high FRA*P* value that implies the potent antioxidant property of AEPG.

Nitric oxide radical, plays a crucial role in the many biological reactions. If generated in excess, it reacts with superoxide to form peroxynitrite. At physiological pH, this peroxynitrite gets rapidly protonated to form peroxynitrous acid, a powerful oxidizing and nitrating agent which may damage proteins, lipids, and DNA of the body, or it may have degraded to nitrogen dioxide and hydroxyl radical (most reactive FR) responsible for the cytotoxic action of nitric oxide. Therefore, scavengers of the excess NO can be useful in the prevention of disease caused by OS.[1,26] In our study, we observed that AEPG showed lowest IC_{50} values in nitric oxide scavenging assay than quercetin and ascorbic acid. It suggests that AEPG contains constituents, which can scavenge the cytotoxic NO, and its high antioxidant potency. It is further supported by highest correlation (r = 0.9966) between the nitric oxide scavenging assay and TPC and TFC.

Hydrogen peroxide is formed in the peroxisomes and also when superoxide is reduced by SOD. Glutathione peroxidase removes H_2O_2 by forming water and oxidizing reduced GSH. Catalase (CAT) decomposes H_2O_2 to water and oxygen. However, if formed in excess, it forms hydroxyl radical when it reacts with superoxide in the presence of Fe^{2+}. Hydroxyl radical is the powerful oxidizing agent, which damages the cellular components including sugars and bases of DNA.[1,2] Our study demonstrated significant correlation between TPC, TFC and H_2O_2 scavenging activity (r = 0.9748).

Hydroxyl radical (HO*) is the potent, short-lived highly toxic FR which instigates lipid peroxidation leading to disruption of biological membrane integrity and its function and also causes DNA damage. Hydroxyl radical is formed when superoxide radical reacts with hydrogen peroxide in

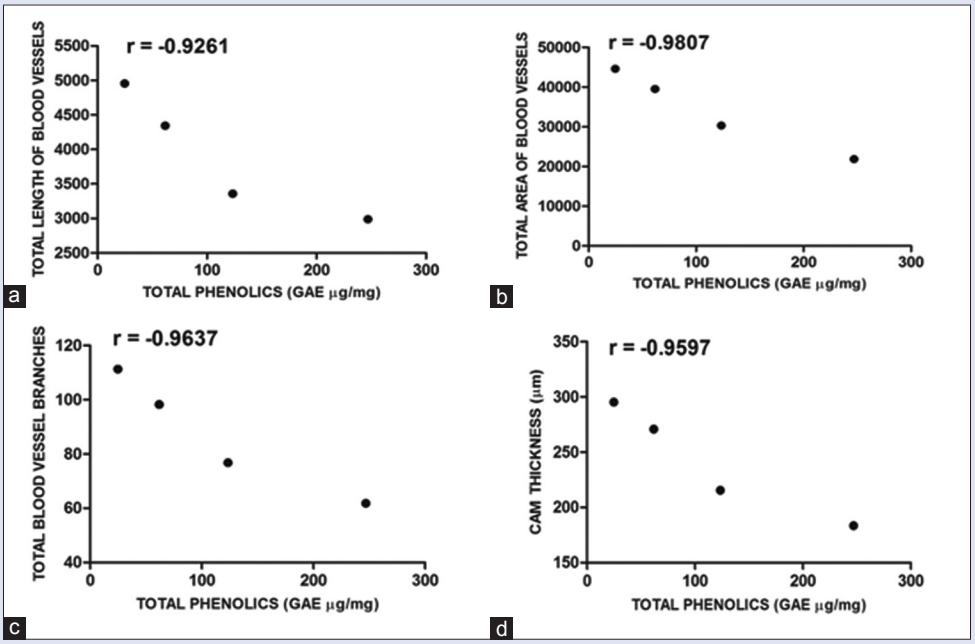

Figure 7: Pearson's correlation analysis between total phenolic content of aqueous extract of *Psidium guajava* (50, 125, 250, 500 µg/ml) and anti-angiogenic activity. (a) total phenolic content versus total length of the blood vessels* (b) total phenolic content versus total area of the blood vessels** (c) total phenolic content versus total branches of blood vessels* (d) total phenolic content versus chorioallantoic membrane Thickness* (*r* = Pearson's Correlation Coefficient) (*$P < 0.05$, **$P < 0.01$)

the presence of Fe^{2+}.[1,2,26] It is important to mention that there is no an endogenous scavenger or enzymes available to scavenge the hydroxyl radical. Therefore, natural plant-based antioxidants are widely used for the prevention of cancer and aging, to scavenge the hydroxyl radical. Our study showed strong correlation between TPC, TFC and hydroxyl RSA ($r = 0.992$).

Superoxide radical is endogenously produced within the body by mitochondrial electron transport chain and in neutrophils by NADPH oxidase etc. SOD is an enzyme found in all the cells, involved in superoxide radical scavenging through the formation of oxygen and hydrogen peroxide. In case of overproduction of superoxide, and/or when SOD is depleted, it reacts with NO to form toxic peroxynitrite or it reacts with H_2O_2 in the presence of Fe^2 + to form highly reactive hydroxyl radical.[1,2] Our study showed significant correlation between TPC, TFC, and superoxide RSA ($r = 0.979$). Similarly, our results are in line with a previous study reported a strong positive correlation between superoxide RSA and TPC ($r = 0.927$) and ABTS ($r = 0.991$) in the leaves and fruits extracts of *P. guajava*.[40]

Angiogenesis is the multistep process requires angiogenic growth factors which bind with the receptors present on the ECs of the preexisting blood vessels, which in turn releases proteases that degrade the basement membrane of ECs to escape and then migrate and proliferate in the surrounding matrix and form new blood vessels.[6,7] Although many methods are available to evaluate the angiogenic/antiangiogenic activity, gelatin sponge– CAM assay method is preferred as it is very simple, easy to perform, inexpensive, does not require sophisticated instruments; reliable, and large samples can be screened in short period.[35,41] Ribatti *et al.* presented a screening method for anti-angiogenic activity in which test compound had to be mixed with one of the known angiogenic factors such as VEGF, FGF-2 in a fixed concentration. Accordingly, in our study, we validated the VEGF concentration and used 10 ng of VEGF per egg.[35,41]

Quercetin 20 µM and 40 µM per egg (reported earlier) were utilized[42] in our study to compare the anti-angiogenic activity of AEPG. The concentration of quercetin present in the AEPG was quantified using LC-MS and also revealed the presence of guavanoic acid, luteolin, myrecetin and kaempferol were already reported.[43] In another study, it was observed that almost similar constituents present in the AEPG were reported using LC-ESI/MS.[18]

Our data showed significant reduction in total length, area, and branches of blood vessels and CAM thickness of V10 + AEPG 125 µg and V10 + Quercetin 20 µM groups, V10 + AEPG 250 µg and V10 + Quercetin 40 µM groups exhibited almost similar results, respectively; however, there was no significant reduction in V10 + AEPG 50 µg. The V10 + AEPG 500 µg group showed a maximum reduction in the total length, area, branches of blood vessels and CAM thickness. In the present study, the total phenolic and flavonoid content showed statistically significant correlation with antioxidant and radical scavenging activity of AEPG and also the TPC of AEPG shown statistically significant correlation with reduction in total length, area, branches of blood vessels, and CAM thickness in AEPG treated groups. These data collectively imply the concentration-dependent antiangiogenic activity of AEPG may be due to its phenolic and flavonoid contents.

Quercetin has been shown as a potent natural anti-angiogenic flavonoid. Researches proposed many mechanisms, which may be responsible for its anti-angiogenic action. Peng and his group's study was the first and only reported antiangiogenic activity of AEPG using CAM, however, they used a single very high dose, of 5 mg per egg.[44] However, our study is the first one to show the concentration-dependent effect of AEPG on antiangiogenic activity using chick CAM.

Nearly 80% of the world populations rely on the herbal medicines for their primary healthcare.[45] In traditional medicine, herbal drugs have been widely used in the treatment of various diseases as plant drugs offer better therapeutic effects with minimal side effects, may have synergistic effect by acting on different targets or it can improve the pharmacokinetic property.[46] The study reveals that AEPG can be used in the prevention of cancer, obesity, psoriasis, diabetic retinopathy, asthma, inflammatory bowel disease, liver cirrhosis, arthritis, and diabetic nephropathy due to its antioxidant and antiangiogenic property.

CONCLUSION

The present study demonstrated potent antioxidant activity of AEPG due to the presence of significant amount of polyphenolic and flavonoids contents. The AEPG exhibited concentration-dependent antiangiogenic activity and displayed a significant correlation with its TPCs. Therefore, AEPG can be potentially used as chemopreventive in ROS implicated diseases and pathological angiogenesis. However, further studies are needed to prove the molecular mechanism of its action.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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