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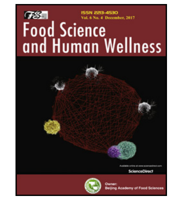

# Phenolics, tannins, flavonoids and anthocyanins contents influenced antioxidant and anticancer activities of Rubus fruits from Western Ghats, India

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ABSTRACT

*Rubus* fruits are rich in nutrition with a wide range of phytochemical and consumed by humans, but many of its wild varieties are not commercially useful. Phytochemical exploration of these wild fruits could improve the commercial usage of these *Rubus* species.

This study focuses to analyze the phytochemical, antioxidant and cytotoxicity of three wild *Rubus ellipticus*, *R. niveus* and *R. fairholmianus* fruits comparatively, which has not revealed. The *in vitro* antioxidant studies indicated the ethyl acetate and methanol extracts of studied *Rubus* species competent in scavenging different stable and generated radical. The correlation and regression analysis also statistically supported the contents in the extracts could act against various radicals. Correlation between phytochemical constituent and radical scavenging activity found to be highly positive. The results acquired from this investigation revealed that they are equally competent and could be recommended as dietary supplements or as nutraceuticals.



## 1. Introduction

Wild edible fruits add appreciably to the nutrition of tribal people, although these fruits are taken by people in raw as well as in processed forms. However, a transition in the nutritional diet is going on around the world by replacing animal fats diets with traditional plant product diets [1]. Apart from their medicinal and nutritional content, these wild edible fruits also have significant trade economical advantages [1]. Adding scientific conclusions related to the health benefits of these wild edible fruits would be an effective addition to the wild plants. The underutilized wild edible fruits are more nutritious than the other consumable fruits available in the market, but we often failed to utilize natural products. Among them, berries are known for their rich phytoconstituents such as phenolics, flavonoids and procyanidins with extensive therapeutic purposes for the better health status of human beings [1]. Addition of these fruits in the diet has a strong effect against heart disease, diabetes, and cancer. The phenolic and flavonoid compounds present in these underutilized fruits are known to act as antioxidants [2–5]. Particularly, in the last few decades studies proved the role of utilization of natural products rich in antioxidant contents including vitamins, phenolics, flavonoids and carotenoids against oxidative diseases [2]. These polyphenolic compounds in edible fruits can effectively scavenge reactive oxygen species (ROS) and prevent the cells from oxidative damage, which leads to cancer and cardiovascular disorder [3]. Thus, the researches examining the antioxidant capacity of fruits are increasing when compared to the past. This fruits could use as functional foods also in the pharmaceutical, food and beverage industries [4,5].

*Rubus*, the largest genus (Family: Rosaceae) which well known as highly nutritive wild edibles like brambles and raspberries. *R.*

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*ellipticus* is used mainly among tribal's for gynecological problems, diarrhea, curing bone fracture, relieving stomach worms in children, antifertility activity in males and also for edible purposes [6,7]. Rural populations use *R. fairholmianus* leaves and fruits as digestible, for curing headache, as a stimulant and besides as insecticides [5–7]. Moreover, *R. niveus* fruits are consumed by many of the tribal sections of our country, for control of excessive bleeding in women during menstrual problem [7,8].

Though various researchers have done in the *Rubus* fruits, this study carried out in the fruits of *R. niveus*, *R. ellipticus* and *R. fairholmianus* as a comparative analysis to estimate and evaluate their phenolic, tannin, flavonoid and anthocyanin content's relationship with the antioxidant, anticancer activities using regression analysis. This study can explain the role of *Rubus* fruits as potentially antioxidant supplements in the daily diet and as ingredients in pharmacological industries.

## 2. Materials and methods

### 2.1. Collection and identification of plant materials

The fruits of *R. fairholmianus*, *R. niveus* and *R. ellipticus* collected from Shola forest of Marayoor, Kerala, India (10.2762° N, 77.1615° E) in the month of December 2013. The plant taxonomic identity confirmed by Botanical Survey of India, Southern Circle, Coimbatore, Tamilnadu, India. The surface pollutants from the collected fruits washed by distilled water rinsing. The shade dried fruit homogenized into a fine powder using the blade grinder (Preethi Kitchen Appliances Pvt. Ltd; Chennai, India).

### 2.2. Chemicals, solvents and reagents

The analytical grade chemicals, solvents and reagents used were purchased from Himedia Laboratories, Mumbai, India; Sisco Research Laboratories, Mumbai, India and Sigma Aldrich, Bengaluru, India. The assays were carried out using Milli-Q water (18.2 MΩ $cm^{-1}$ resistivity; less than 5 ppb total oxidizable carbon) (Merck Millipore; Billerica, USA).

### 2.3. Preparation of Rubus fruits extracts

All the three *Rubus* species fruit powder were packed as independent thimbles using Whatman no 1 filter paper and extracted progressively with petroleum ether, ethyl acetate and methanol by utilizing Soxhlet apparatus (Borosil Glass Works Ltd; Mumbai, India), each time before separating with the following solvents, the thimble was dried in hot air oven (Meta Lab Scientific Industries; Maharashtra, India) at 20 °C. Subsequently, the extracted material has been macerated for 2 days using hot water, and the extract was filtered using Whatman No. 1 filter paper. Finally, the organic solvent and water extracts were concentrated by rotary vacuum evaporator (Equitron; Medica Instrument Mfg. Co; Mumbai, India) and then air dried extracts were weighed and collected in storage containers for further studies. The extracting capacity of each used solvent was determined using Eq. (1),

$$\text{Extract Recovery Percent} = \frac{X}{Y} \times 100 \tag{1}$$

where *X* is quantity of extract of obtained in gram; *Y* is the sum of sample taken for extraction in gram.

### 2.4. Quantification of total phenolic, tannin and flavonoid contents in the Rubus extracts

Folin-Ciocalteu colorimetric method explained by Makkar [9] was followed to decide the total phenolic content (TPC) in the various extracts of *R. fairholmianus*, *R. niveus* and *R. ellipticus* and expressed as mg equivalents of a gallic acid per g of *Rubus* extract. About 200 μL extract was made up to 1 mL with distilled water (0.8 mL) and then mixed with 1 N of 500 μL Folin-Ciocalteau phenol reagent. Then the tubes were incubated for 5 min by adding 5% of 1.250 mL sodium carbonate solution. Following 40 min dark incubation at room temperature, the absorbance was read at 725 nm against a blank reagent.

Polyvinylpolypyrrolidone (100 mg), was added with 500 μL of extract and kept for the incubation of 15 min. At 4 °C for 10 min the test tubes were centrifuged at 4350 × *g*. The non-tannin phenolics containing supernatant was used in the determined as described in the Makkar [9]. The tannin content present in the plant extracts was calculated using Eq. (2):

$$\text{Tannins} = \text{Total phenolics} - \text{Non tannin phenolics} \tag{2}$$

The flavonoids content (FC) was determined using Zhishen et al. [10] method with rutin hydrate (flavonoid glycoside) as reference compound and expressed in the equivalents rutin. About 500 μL of extracts was incubated for 6 min with 2 mL distilled water, followed by at room temperature 150 μL of 5% sodium nitrite, 10% aluminum chloride and 2 mL of 4% sodium hydroxide solutions were added, consequently the volume of the test tube made up to 5 mL with distilled water. After the 15 min incubation samples with pink shading was perused at 415 nm against a blank reagent.

### 2.5. Determination of anthocyanins contents of the extracts

Total anthocyanins estimated using Du et al. [11] pH differential method. The fruit extracts mixed thoroughly with pH 1.0 of 0.025 M potassium chloride buffer (1:2) then mixed with sodium acetate buffer (pH 4.5). Subsequently, at 510 and 700 nm the resultant absorbance read against blank buffer solution of pH 1.0, 4.5 and the absorbance values were converted to total milligrams of cyanidin 3-glucoside (C3G). The anthocyanin content was calculated using Eqs. (3) and (4):

$$\Delta A = (A_{510} - A_{700})_{pH1.0} = (A_{510} - A_{700})_{pH4.5} \tag{3}$$

$$\text{Total monomeric anthocyanins (mg/100g)} = \frac{\Delta a \times MW \times 1000}{(\varepsilon \times C)} \tag{4}$$

where *A* is absorbance, MW (449.2) is the sub-atomic weight of C3G, $\varepsilon$ (26, 900) is the molar absorptivity of C3G and C is the convergence of the extract in mg per mL. The anthocyanin content resulted as milligrams of C3G reciprocals per 100 g of extract.

### 2.6. In vitro free radical neutralizing assays

#### 2.6.1. 2,2-diphenyl-1-picryl hydrazyl radical scavenging activity of Rubus extracts

The hydrogen donating ability of *Rubus* fruit extracts were examined using 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) stable free radical according to the Blois [12] method. Aliquots of sample extracts and butylated hydroxyl toluene (BHT) and rutin with 100 μL volume adjusted with methanol incubated in the dark for 30 min with 3 mL of 0.1 mM DPPH in methanol was added and vortexed well. The neutralized radical content was compared by adding 100 μL of methanol in 3 mL of 0.1 mM methanolic solution of DPPH as the negative control. The scavenging activity of the samples was measured as optical density at 517 nm against the methanol as the blank solution and the results were expressed as the extract required to inhibiting DPPH radical to 50% concentration ($IC_{50}$).

#### 2.6.2. 2,2′-azinobis (3-ethyl- benzothiozoline)-6-sulfonic acid disodium salt radical scavenging activity of Rubus extracts

Total antioxidant activities of the fruit extracts were measured by 2,2′-azinobis (3-ethyl-benzothiozoline)-6-sulfonic acid disodium salt cation radical ($ABTS^{\bullet+}$) decolourization assay. The radicals were generated by incubating 7 mM ABTS aqueous solution for 12–16 h in dark with 2.4 mM of potassium persulfate at room temperature. Based on the Re et al. [13] method, the incubated solution was further diluted to 1:89 v/v in ethanol and the absorbance was calibrated to 0.700 at 734 nm. About 1 mL of finally diluted ABTS solution was incubated in dark for 30 min at room temperature with extracts (50 μL) and trolox (0–15 μM). The absorbance value at 734 nm against the ethanol was used to calculate the total antioxidant activity and expressed as μM trolox equivalents/g sample extracts.

#### 2.6.3. Ferric reducing antioxidant power of Rubus extracts

The ferric reducing antioxidant power (FRAP) of *Rubus* spp fruit extracts were estimated accordingly by Pulido et al. [14]. The freshly prepared FRAP reagent of 900 μL was incubated for 30 min under 37 °C with distilled water (90 μL), 30 μL of test sample and methanol for blank reagent. While the FRAP reagent was prepared by mixing 2.5 mL of 20 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 20 mM ferric chloride hexahydrate each mixed with 25 mL of 0.3 M acetate buffer with pH-3.6. The optical density was read at 593 nm against the reagent blank in the spectrophotometer (Shimadzu, Kyoto; Japan). The calibration curve was prepared to calculate the reducing ability of the extract using known concentration ferrous (Fe II) in methanol (100 to 2000 μM) and the antioxidant power was expressed in ferric-TPTZ reducing power corresponding to that of 1 mM ferrous sulfate heptahydrate.

#### 2.6.4. Phosphomolybdenum reduction assay of Rubus extracts

The ability to forming a green phosphomolybdenum complex [15] of *Rubus* fruits extracts was evaluated by phosphomolybdenum reduction assay (PRAA). A triplicate of 100 μL of test sample and ascorbic acid (1 mM) in distilled water (blank) was mixed with 3 mL of PRAA reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the test tubes incubated at 95 °C for 90 min. At room temperature, the OD was read at 695 nm with blank reagent and the PRAA results were calculated as ascorbic acid equivalents.

### 2.7. High performance liquid chromatography analysis of Rubus extracts

The analysis were performed using an Shimadzu preparative/analytical HPLC instrument (Shimadzu Corp, Kyoto, Japan) equipped with a binary solvent pump (LC 20 AP) coupled to a diode array detector (DAD) (SPD N 20A) with C18 column (5 μm; Dimension-4.6 × 250 mm). The chromatogram was processed using LabSolutions$^{TM}$ software (Shimadzu Corporation, Kyoto, Japan). The analytical procedure was performed applying the chromatographic conditions previously described by Muniyandi et al. [16]. The standard compound kaempferol was prepared using water and filtered by 0.45 μm PVDF membrane and a volume of 20 μL was injected.

Water (solvent A) and water: acetonitrile: acetic acid (47:50:3 v/v) (solvent B) were used as mobile phase and a gradient elution program was created with 100% of solvent A at 0.1 min as initial concentration then gradually solvent B was increased to 35% in 25 min, 50% attained in 45 min and finally 100% at 65 min. The rate of flow was maintained at 1 mL/min and 20 μL of sample was injected for the analysis. The reference compounds were analyzed discretely to get the retention time.

#### 2.7.1. Linearity

The linearity range was obtained by repeated injection of five different concentrations of a standard in HPLC grade water. An analytical curve of each standard compound was obtained based on the correlation among the peak area and the individual standard concentration using a linear model.

#### 2.7.2. Determination of detection limits and method quantification

To acquire the limits of detection (LOD) and quantification (LOQ), the strategy depicted by Hubaux and Vos [17] was utilized. LOD and LOQ (Eqs. (5) and (6)) were calculated from a standard analytical curve of different standard concentrations.

$$\mathrm{LOD} = 3.3 \times \frac{\sigma}{S} \quad (5)$$

$$\mathrm{LOQ} = 10 \times \frac{\sigma}{S} \quad (6)$$

where $\sigma$ – standard deviation of the response; $S$ – standard calibration curve slope.

### 2.8. Anticancer activity of Rubus extracts

The human colon cancer cell line (Caco-2; ATCC accession number HTB-37) was procured from the National Center for Cell Sciences, Pune, India and maintained in minimal eagle media supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and streptomycin antibiotics in 5% $CO_2$ at 37 °C in a humidified incubator.

The anticancer property of the *Rubus* fruits extracts was determined using Caco-2 cell lines using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with slight modifications from our previous studies [16,18]. Using MEM excluding FBS and 0.01% Dimethyl sulfoxide (DMSO), the methanol extracts of *R. fairholmianus*, *R. niveus* and *R. ellipticus* were dissolved and diluted to working concentration ranging from 1–10 μg/μL in the ratio of 1:1. After 12 h, the seeded $1 \times 10^5$ cells/well in 96-well plates were incubated for 24 h with various concentrations of the extract. The plate well was replaced and incubated for 3 hr at 37 °C with 100 μL of MTT in phosphate buffered saline solution (5 mg/mL). The OD of formazan crystal dissolved in DMSO (100 μL) was read at 570 nm using multiplate reader (Tecan infinite 200 pro; Männedorf, Switzerland). The morphological changes of the cells were imaged under an inverted magnifying instrument (Olympus; Shinjuku-ku, Japan). The cell feasibility was expressed as a level of control and calculated utilizing condition 7:

$$\%\,\text{Cell viability} = 100 - [100 \times (X - Y) \div X] \quad (7)$$

where $X$ is the OD value of the control and $Y$ is the % Cell viability value of extracts.

### 2.9. Statistical analyses

Every experiment was done in triplicates and the outcomes were expressed as Mean ± Standard Deviation. The informations were factually investigated utilizing one way ANOVA has taken after by Duncan's test and the relationship amongst phytocompounds and antioxidant activity were analyzed in correlation and regression strategy utilizing SPSS programming (Version 20.0. SPSS Inc., Chicago, USA).

## 3. Results and discussion

Recently, the hot percolation method of extracting plant samples using Soxhlet extractor became most popular, simple and economic [16]. The extracting capacity of the used organic solvents

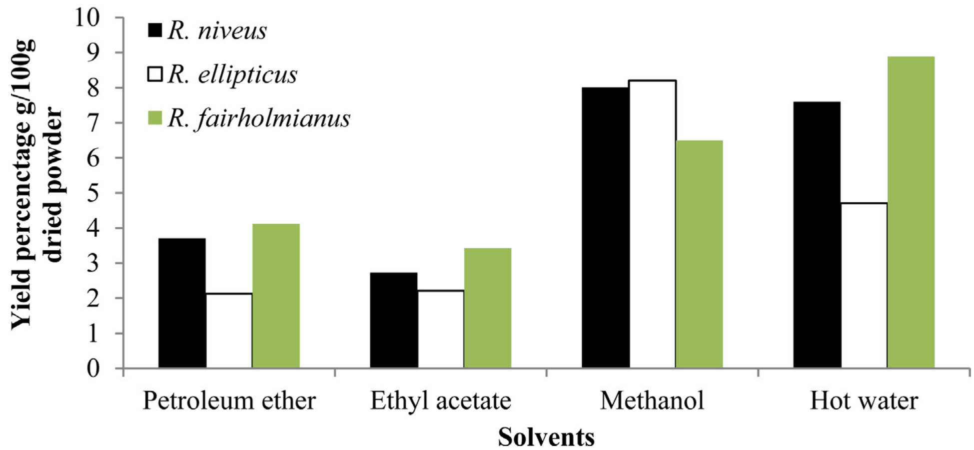


**Fig. 1.** Extract yield of *Rubus* spp using various solvents.

**Table 1**
Total phenolic, tannin, flavonoid and anthocyanin contents of *Rubus* spp fruit extracts.

| Samples | TPC (mg GAE/g) | TAC (mg TAE/g) | FLC (mg RE/g) | ANC (mg/100g) | DPPH ($IC_{50}$-μg/mL) | ABTS (μM TE/g) | PRAA (mg AAE/g) | FRAP (mM Fe(II)/mg) |
|---|---|---|---|---|---|---|---|---|
| RNPE | 10.19 ± 0.63 | 18.65 ± 0.52 | 47.08 ± 0.83 | 0.12 ± 0.10 | 352.12 ± 0.35 | 1125.32 ± 94.40 | 147.04 ± 21.53 | 0.82 ± 0.03 |
| RNEA | 147.86 ± 0.96 | 242.83 ± 2.39 | 108.33 ± 1.67 | 1.93 ± 0.09 | 17.32 ± 0.31$^{b}$ | 7777.33 ± 404.15 | 170.36 ± 18.45$^{c}$ | 22.68 ± 0.52 |
| RNME | 361.02 ± 0.96$^{b}$ | 565.11 ± 4.90$^{b}$ | 184.72 ± 1.73$^{b}$ | 4.09 ± 0.06$^{a}$ | 12.48 ± 0.30$^{b}$ | 16,247.17 ± 344.45$^{b}$ | 197.92 ± 4.30$^{bc}$ | 49.56 ± 0.40$^{b}$ |
| RNHW | 28.79 ± 1.10 | 20.21 ± 1.55 | 79.17 ± 1.25 | 0.18 ± 0.01 | 18.31 ± 0.20$^{b}$ | 8869.36 ± 528.52 | 239.28 ± 1.24$^{a}$ | 22.42 ± 3.52 |
| REPE | 17.40 ± 0.63 | 17.62 ± 1.87 | 44.31 ± 1.46 | 0.08 ± 0.03 | 272.79 ± 0.61 | 732.45 ± 115.33 | 130.37 ± 15.01 | 0.44 ± 0.03 |
| REEA | 143.68 ± 0.96 | 230.40 ± 1.58 | 157.78 ± 1.73$^{c}$ | 0.30 ± 0.07 | 26.63 ± 0.32 | 5846.32 ± 294.49 | 196.98 ± 1.29$^{bc}$ | 23.05 ± 1.35 |
| REME | 401.36 ± 0.96$^{a}$ | 628.32 ± 3.17$^{a}$ | 215.00 ± 2.20$^{a}$ | 3.18 ± 0.10$^{c}$ | 11.01 ± 0.36$^{b}$ | 18,804.10 ± 234.10$^{a}$ | 215.13 ± 5.95$^{ab}$ | 57.05 ± 0.71$^{a}$ |
| REHW | 30.36 ± 0.48 | 11.05 ± 0.30 | 102.64 ± 1.46 | 0.19 ± 0.03 | 23.94 ± 0.64 | 8762.82 ± 441.89 | 172.12 ± 4.03$^{c}$ | 24.35 ± 1.72 |
| RFPE | 9.46 ± 0.18 | 7.77 ± 1.55 | 31.53 ± 1.05 | 0.36 ± 0.06 | 406.77 ± 0.75 | 1291.78 ± 189.86 | 136.03 ± 1.91 | 0.44 ± 0.04 |
| RFEA | 35.84 ± 1.31 | 42.14 ± 4.31 | 81.67 ± 2.20 | 0.43 ± 0.07 | 109.83 ± 0.15 | 4141.70 ± 400.19 | 145.22 ± 1.69 | 4.84 ± 0.10 |
| RFME | 201.57 ± 0.63$^{c}$ | 306.18 ± 1.33$^{c}$ | 102.22 ± 1.27 | 3.65 ± 0.06$^{b}$ | 23.89 ± 0.16 | 8802.77 ± 362.52 | 169.04 ± 7.48$^{c}$ | 32.01 ± 0.37 |
| RFHW | 34.54 ± 0.48 | 8.29 ± 1.37 | 39.58 ± 1.44 | 1.15 ± 0.08 | 21.19 ± 0.26 | 12,145.43 ± 312.04$^{c}$ | 236.97 ± 0.25$^{a}$ | 37.67 ± 0.90$^{c}$ |
| *Standard compounds used in the antioxidant studies* | | | | | | | | |
| Rutin | | | | | 6.35 ± 0.40$^{a}$ | 9942.69 ± 29.34 | 437.19 ± 6.85 | 99.19 ± 14.90 |
| Butylated hydroxytoluene | | | | | 7.93 ± 0.40$^{a}$ | 8423.95 ± 22.31 | 443.51 ± 6.43 | 112.56 ± 9.58 |

TPC – Total phenolic content; TAC – tannins content; FLC – flavonoids content; ANC – anthocyanin content; DPPH – 2,2-diphenyl-1-picryl hydrazyl radical scavenging activity; ABTS – 2,2′-azinobis (3-ethyl-benzothiozoline)-6-sulfonic acid disodium salt scavenging activity; PRAA – Phosphomolybdenum reduction antioxidant assay; FRAP – Ferric reducing antioxidant power assay; GAE – Gallic Acid Equivalents; TAE – Tannic Acid Equivalents; RE – Rutin Equivalents; AAE – ascorbic acid equivalents; TE – trolox equivalents; $IC_{50}$ – inhibition concentration 50; RNPE – *Rubus niveus* petroleum ether; RNEA – *Rubus niveus* ethyl acetate; RNME – *Rubus niveus* methanol; RNHW – *Rubus niveus* hot water; REPE – *Rubus ellipticus* petroleum ether; REEA = *Rubus ellipticus* ethyl acetate; REME – *Rubus ellipticus* methanol; REHW – *Rubus ellipticus* hot water; RFPE – *Rubus fairholmianus* petroleum ether; RFEA – *Rubus fairholmianus* ethyl acetate; RFME – *Rubus fairholmianus* methanol; RFHW – *Rubus fairholmianus* hot water; Values are mean of triplicate determination ($n = 3$) ± standard deviation; Statistically significant at $p < 0.05$ where $^{a>b>c}$ in each column.

was described in terms of yield percentage were shown in Fig. 1. The higher recovery percentage in hot water and methanol reveals that the plant might be contributed to the phenolic compounds in the respected extracts [18]. The amount of phenolics content in ethyl acetate and methanolic extracts ranged from 401.36 to 35.84 mg GAE/g of extract in the order of *R. ellipticus* > *R. niveus* > *R. fairholmianus* (Table 1). Among all, *R. ellipticus* methanol extract showed the highest amount of total phenolic content (401.36 mg GAE/g extract) (Table 1). The phenolic, tannins, flavonoids and anthocyanin's contents were found to be higher on methanol extract of *R. ellipticus*, *R. niveus* and *R. fairholmianus* which indicates the importance of these fruit plants in terms of possesses bioactive compounds and are used in different health-related activities [19] due to their established role in absorbing and neutralizing the free radicals and are the main constituents of human diet. The methanol extracts of *R. ellipticus* and *R. niveus* fruits (628.32 and 565.11 mg TAE/g of extract) showed higher tannins content (Table 1). Chung et al. [20] related antioxidative property with anticarcinogenic, antimutagenic potentials of high molecular weight phenolics, which is important in protecting cellular oxidative damage. Flavonoids are ubiquitous dietary chemicals which significant influence *in vivo* effects within the immune system comprising the inflammatory response [21]. In the case of flavonoid contents the methanol extracts of *R. ellipticus* (215.00 mg RE/g of extract) resulted in higher amount when compared to other extracts followed by methanol extracts of *R. niveus* (184.72 mg RE/g of extract), and ethyl acetate extract of *R. ellipticus* (157.78 mg RE/g of extract) (Table 1). The anthocyanin's quantification revealed higher content in *R. niveus* methanolic extract (4.09 mg anthocyanin/100 g) whereas the methanolic extracts of *R. fairholmianus* and *R. ellipticus* were comparatively lower (3.65 and 3.18 mg anthocyanin/100 g extract) (Table 1). Therefore, the higher amounts of phenolics can enhance the biological property of wild edible fruits.

Concerning the higher amount of phytocompunds, the *R. niveus*, *R. ellipticus* and *R. fairholmianus* extracts proposed superior radical scavenging activities (Table 1). The results were justifying the presence of more biological active phenolics, tannins and flavonoids [12]; the methanol extracts of *R. ellipticus* and *R. niveus* showed improved hydrogen donating ability against stable DPPH radical compared to other solvent extracts (11.01 and 12.48 μg/mL respectively). ABTS assay could be used to determine the trolox based antioxidant assay of natural products [13]. The methanol extracts of *R. ellipticus* and *R. niveus* fruit showed 18,804.10 and 16,247.17 μM TE/g of extract activity in ABTS radical scavenging activity (Table 1).

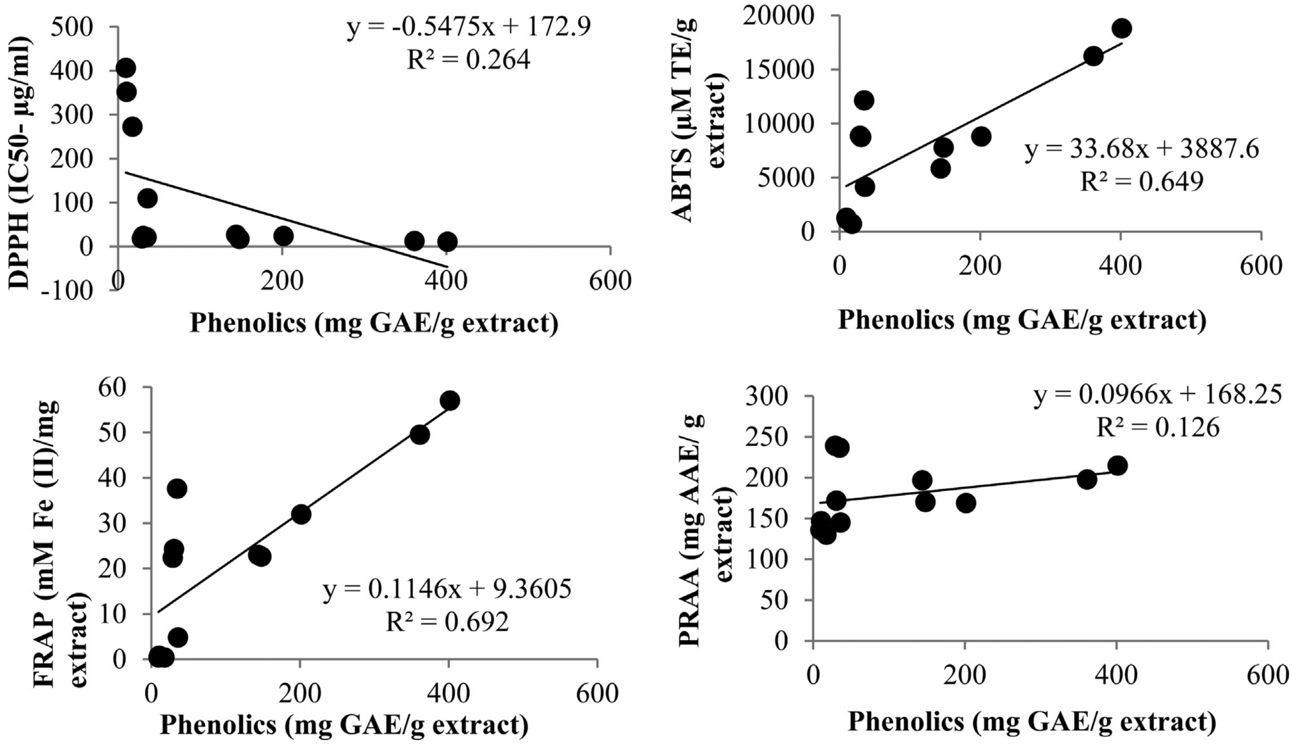


**Fig. 2.** Relationship between phenolics content and antioxidant activities of *Rubus* spp.

Hagerman et al. [22] revealed phytocompounds containing high molecular weight with a benzene ring can quench free radicals more effectively. Prieto et al. [15] emphasized phosphomolybdenum reduction method is characteristically applied to evaluate the antioxidant potential of plant products. *R. fairholmianus* hot water extract of showed better molybdenum reducing the ability with 236.97 mg AAE/g of extract (Table 1), and the FRAP assay revealed methanol extract of *R. ellipticus* (57.05 mM Fe (II)/mg extract) was potential to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) (Table 1). These results implied the exploitation of wild edible fruit's species with high antioxidant could be valuable in the daily dietary supplement [23,24] and thus *Rubus* fruits can perform well as potent antioxidant foods.

The antioxidant capacity of *Rubus* fruits can be related to its phenolics, flavonoids, tannins and anthocyanin contents [25]. Correlations and regression analysis were done between phenolics, flavonoids, tannins, anthocyanin's contents and antioxidant activity obtained from *Rubus* spp. All the antioxidant assays showed relatively positive correlation between all the estimated phytocomounds. Particularly DPPH assay showed negative correlation and regression with the phytocompounds because the related antioxidant activity calculated in $IC_{50}$ value where higher amount indicating lower radical scavenging activity and *vice versa* (Table 2; Figs. 2–5). Among the estimated phytocompounds flavonoid contents highly interrelated with DPPH radical scavenging activity ($r = -0.629$, $R^2 = 0.395$, $P = 0.029$) (Table 2). Interestingly all the estimated compounds were positively associated with the antioxidant activity of the *Rubus* fruit's extracts, particularly phenolics and tannins were highly influenced the ABTS (Phenolics – $r = 0.806$, $R^2 = 0.649$, $P = 0.002$; Tannins – $r = 0.768$, $R^2 = 0.589$, $P = 0.004$ respectively) and FRAP (Phenolics – $r = 0.832$, $R^2 = 0.692$, $P = 0.001$; Tannins – $r = 0.797$, $R^2 = 0.635$, $P = 0.002$ respectively) (Table 2; Figs. 2–5) antioxidant activity significantly. Whereas the flavonoids and anthocyannins contents were also significantly correlated and influenced the antioxidant activity with $r = 0.753$, $R^2 = 0.567$, $P = 0.005$; $r = 0.740$, $R^2 = 0.548$, $P = 0.006$ for ABTS and $r = 0.772$, $R^2 = 0.596$, $P = 0.003$; $r = 0.776$, $R^2 = 0.602$, $P = 0.003$ for FRAP respectively (Table 2; Figs. 2–5). These correlations indicate the presences of water soluble antioxidants are most abundant in fruits [26]. This high positive correlation also reported in fruit juices and peaches and plums between antioxidant capacities determined

**Table 2**
Relationship analysis of phenolics, tannins, flavonoids and anthocyanins on antioxidant activity of *Rubus* spp fruits.

| Characteristics | Correlations and Regression analysis | | | | |
|---|---|---|---|---|---|
| | *r* | $R^2$ | *df* | *F* | Significance |
| *Phenolics* | | | | | |
| DPPH | −0.514 | 0.264 | 11 | 3.590 | 0.087 |
| ABTS | $0.806^{**}$ | 0.649 | 11 | 18.489 | 0.002 |
| FRAP | $0.832^{**}$ | 0.692 | 11 | 22.442 | 0.001 |
| PRAA | 0.356 | 0.126 | 11 | 1.447 | 0.257 |
| *Tannins* | | | | | |
| DPPH | −0.480 | 0.231 | 11 | 2.997 | 0.114 |
| ABTS | $0.768^{**}$ | 0.589 | 11 | 14.357 | 0.004 |
| FRAP | $0.797^{**}$ | 0.635 | 11 | 17.375 | 0.002 |
| PRAA | 0.315 | 0.099 | 11 | 1.103 | 0.318 |
| *Flavonoids* | | | | | |
| DPPH | $-0.629^{*}$ | 0.395 | 11 | 6.538 | 0.029 |
| ABTS | $0.753^{**}$ | 0.567 | 11 | 13.095 | 0.005 |
| FRAP | $0.772^{**}$ | 0.596 | 11 | 14.735 | 0.003 |
| PRAA | 0.274 | 0.162 | 11 | 1.931 | 0.195 |
| *Anthocyanins* | | | | | |
| DPPH | −0.483 | 0.233 | 11 | 3.041 | 0.112 |
| ABTS | $0.740^{**}$ | 0.548 | 11 | 12.128 | 0.006 |
| FRAP | $0.776^{**}$ | 0.602 | 11 | 15.104 | 0.003 |
| PRAA | 0.274 | 0.075 | 11 | 0.814 | 0.388 |

DPPH – 2, 2-diphenyl-1-picryl hydrazyl radical scavenging activity; ABTS – 2,2′-azinobis (3-ethyl-benzothiozoline)-6-sulfonic acid disodium salt scavenging activity; PRAA – phosphomolybdenum reduction antioxidant assay; FRAP – ferric reducing antioxidant power assay.

** Correlation is significant at the 0.01 level.

* Correlation is significant at the 0.05 level; *r* – Pearson correlation coefficient; $R^2$ – coefficient of determination; *df* – degrees of freedom.

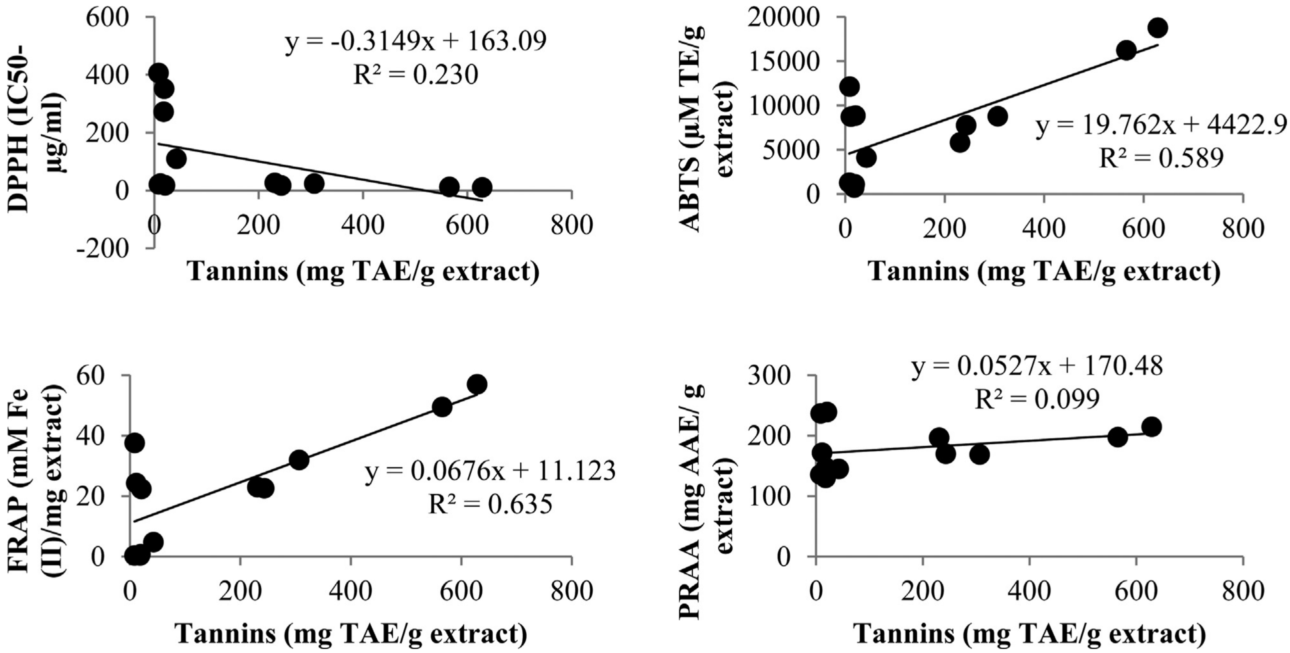


**Fig. 3.** Relationship between tannins content and antioxidant activities of *Rubus* spp.

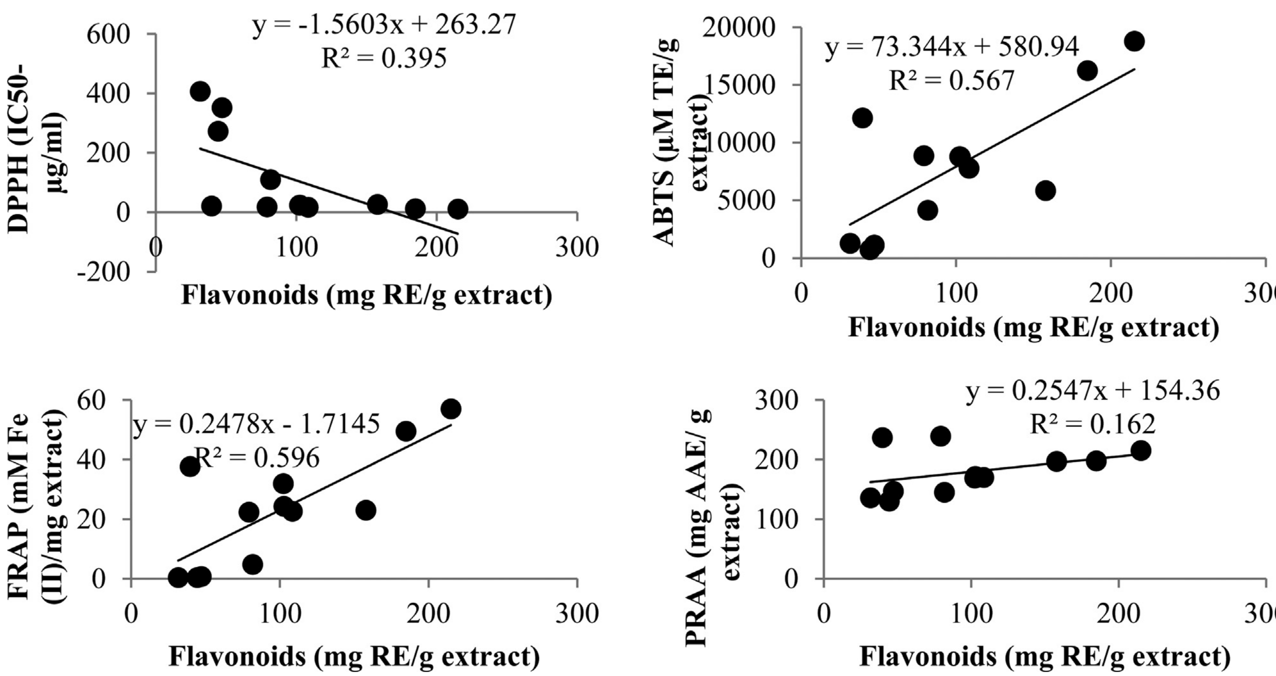


**Fig. 4.** Relationship between flavonoids content and antioxidant activities of *Rubus* spp.

by DPPH, ABTS and FRAP assays [27,28]. Correlation among phytochemical and antioxidant activity was high positive, and the highest correlation was found 0.832 between FRAP and phenolics and the lowest correlation was between PRAA and anthocyanin's content (0.274). It is clear that these phytocompounds were the dominant compounds of the *Rubus* species. The present study results are in agreements with the previous results [29], which concluded that polyphenolic compounds were the major antioxidants present in the *Rubus* fruits.

The HPLC analysis also supported the antioxidant and anticancer activity of *Rubus* extracts due to the presence of kaempferol (Table 3). As depicted in Figs. 6 and 7 the *Rubus* spp methanol extracts significantly controlled the viability of Caco-2 cells based on the dosage used. For a 10 μg/μL concentration it was found that only 50% of Caco-2 cells were viable. Zhong et al. [30] reported that *Rubus* fruits containing more phytochemicals with higher antioxidant activity will ultimately support the antitumor activity. In this way, the cell reinforcement movement of *Rubus* natural products concentrates could add to its higher antitumor action against human colon cancer.

## 4. Conclusions

In conclusion, the nutritional value of fruits alone can bring *Rubus* spp. to the position where functional food meets the demand. The comparative analysis of nutritional properties in the fruits of

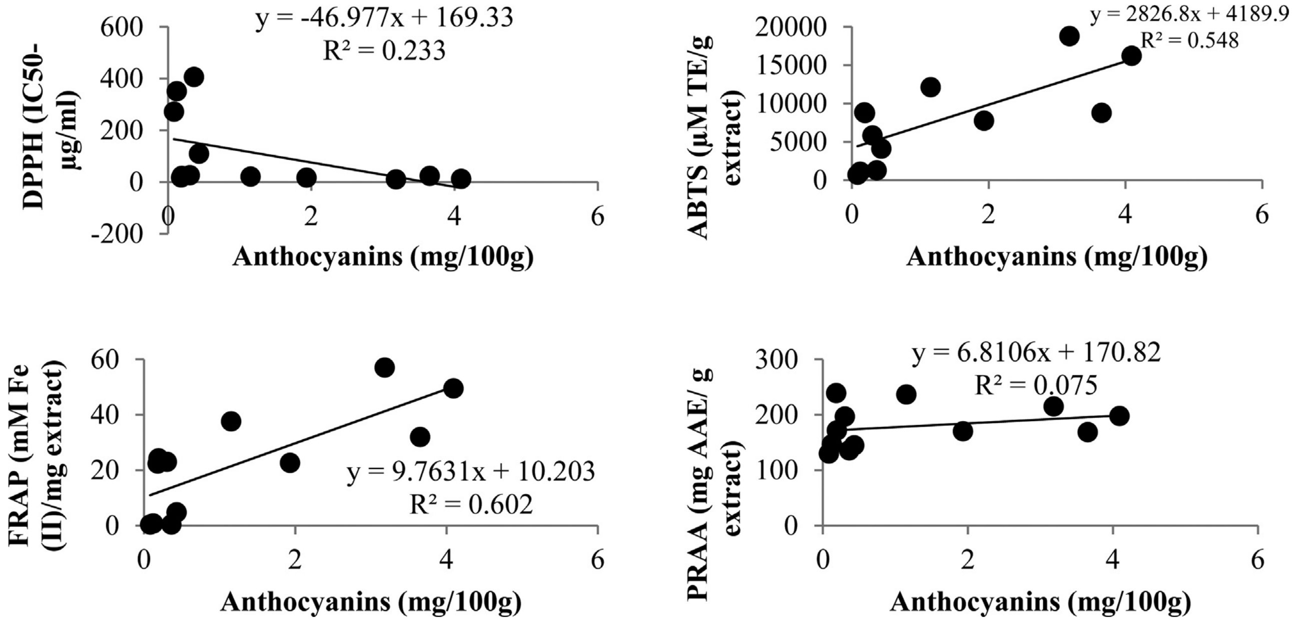


**Fig. 5.** Relationship between anthocyanin contents and antioxidant activities of *Rubus* spp.

**Table 3**
HPLC validation parameters of kaempferol.

| Standards | RT (min) | Range (µg) | Calibration curve | $R^2$ | RSD | LOD (µg/L) | LOQ (µg/L) | Content (mg/g of extract) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | RNME | REME | RFME |
| Kaemferol | 19.411 | 10–50 | $Y = 1.517X - 4.832$ | 0.9596 | 2.416 | 5.256 | 15.926 | 16.85 | 17.4 | 19.8 |

RT – retention time; $R^2$ – coefficient correlation; RSD – standard deviation; LOD – limit of detection; LOQ – limit of detection; RNME – *Rubus niveus* methanol; REME – *Rubus ellipticus* methanol; RFME – *Rubus fairholmianus* methanol.

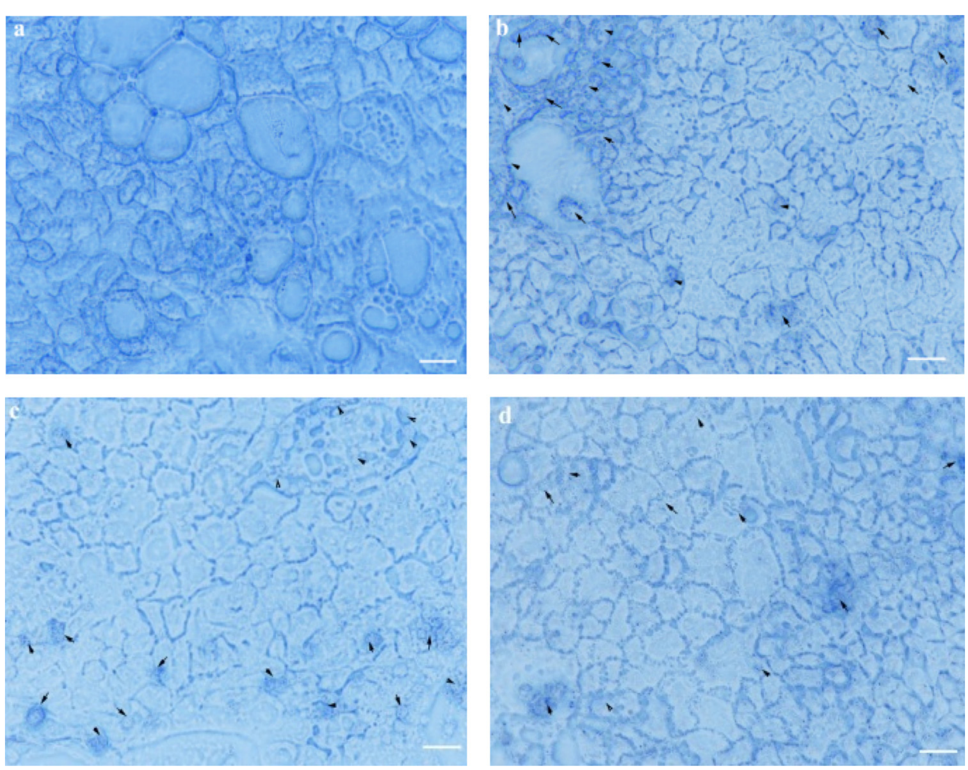


**Fig. 6.** Morphological analysis of cytotoxicity of *Rubus* spp extracts on Caco-2 cells.

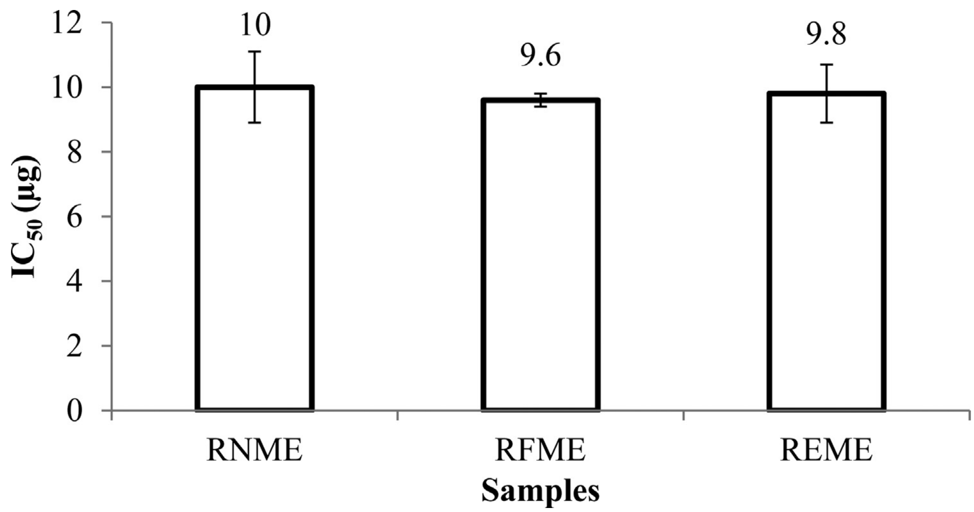


**Fig. 7.** Anticancer analysis of *Rubus* spp fruits methanol extract.

*R. niveus*, *R. ellipticus* and *R. fairholmianus* has been revealed, the methanolic extracts of both *Rubus* spp showed better extracting ability and amount of secondary metabolites. This study is recommending *Rubus* fruits in our diet as they were sure to enhance health and play a key role as a therapeutic to various ailments.

## Conflict of interest

The authors declared there is no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fshw.2019.03.005.

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