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PHYTOCHEMICAL ANALYSIS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *RUBUS APETALUS* POIR. (ROSACEAE)

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Abstract

Rubus apetalus Poir. is a scrambling shrub and belongs to the family Rosaceae. The present study investigates antioxidant activity of leaf extract of *R. apetalus*. Extraction of powdered leaf material was carried out by maceration process using water. Preliminary phytochemical analysis was carried out by standard tests. *In vitro* antioxidant activity was evaluated by four vitro assays viz. DPPH scavenging, ABTS scavenging, hydroxyl radical scavenging and lipid peroxidation inhibition assays. Total phenolic and flavonoid contents were estimated. Phytochemical analysis of leaf extract revealed the presence of saponins, alkaloids, flavonoids, phenolics, tannins, phytosterols and triterpenoids. Leaf extract was found to scavenge DPPH, ABTS and hydroxyl radicals dose dependently with IC_{50} value of 22.49, 17.52 and 33.43µg/ml respectively. Inhibition of lipid peroxidation by leaf extract was also concentration dependent (IC_{50} value 131.63µg/ml). The content of total phenolics and flavonoids were found to be 146.21mg gallic acid equivalents/100g of dry extract and 72.82mg catechin equivalents/100g of dry extract respectively.The plant *R. apetalus* was shown to be an effective in displaying antioxidant potential. The radical scavenging nature of leaf extract indicates the possible utilization of the plant to manage oxidative stress caused by lipid peroxidation and other deleterious effects of free radicals.

Keywords: *Rubus apetalus, Phytochemical, Antioxidant, Free radicals, Lipid peroxidation.*

Introduction

Reactive oxygen species and other reactive species are constantly formed in the body during metabolism and also under certain circumstances such as pollution and exposure to radiations, smoke etc. These free radicals are highly unstable and are known to attack biomolecules such as lipids, proteins and nucleic acids. The consequence is oxidative damage which is implicated in several pathophysiological conditions such as cancer, cardiovascular diseases, neurodegenerative diseases, atherosclerosis and aging. Cells have antioxidant defense system that involves both enzymatic and non-enzymatic systems. Enzymes such as superoxide dismutase, peroxidase and catalase are involved in breaking down and removing free radicals. Non-enzymatic antioxidants act by interrupting free radical chain reactions and include vitamin C, vitamin E, polyphenolic compounds, glutathione and carotenoids. A balance always exists between free radical generation and antioxidant defense in normal healthy person. However, in case of excess generation of radicals and reduced antioxidant defense, the condition leads to oxidative damage. In such situations, there is an extra need for antioxidants from exogenous source mainly in the form of diet. An immense interest in natural antioxidants, mainly from plants, is triggered due to suspected negative effects that are associated with the use of synthetic antioxidants [1-8].

*Rubus apetalus*Poir. (Figure 1) belongs to the family Rosaceae. It is a scrambling shrub reaching up to 2.5 m with hairy branches. The plant is having scattered hooked spines on the stems. Leaves alternate, compounds, leaflets 3-7, about 9 cm long, with a single leaflet at the tip, oval or ellipse-shaped, softly hairy, apex acute, with toothed margins (serrate). Inflorescence panicle, flowers are white to pink, with small or no petals. Fruit consists of many parts (crowded drupes, entire aggregation globose or ovoid), light green, turning yellow to purple-black on ripening. The plant is grown as live fence and for ornamental purpose. Fruits are edible (with sweet acid taste). Roots are medicinal. Plant is visited by honey bees [9-13]. The plant is consumed as food by mountain gorillas in Bwindiimpenetrable national park, Uganda [14]. The fruits of the plant forms diet for Boutourlini's blue monkeys in Ethiopia [15]. *R. apetalus* is consumed as a food plant by geladas (*Theropithecus gelada*) in Guassa community protected area, Ethiopia [16]. The plant *R. apetalus* is used as edible and for the treatment of several ailments. The plant is traditionally used as antivenomous plant in Zaire [17]. In southern Uganda, the plant is used to treat diarrhea and stomachache [18]. In central Kenya, the leaves and fruits of *R. apetalus* were administered orally (after boiling) to treat ENT diseases [19]. Leaves are used to treat syphilis and cough in the Sango bay area, southern Uganda [20]. Fruits are edible and are eaten by herders [12]. In Jimma, Ethiopia, *R. apetalus* is one among the important plants which are used as chewing sticks to meet oral healthcare [21]. In Buhozi site, DR Congo, *R. apetalus* is medicinally used for treatment of mental disorders and infertility [22]. In Hula district of Sidama zone, Ethiopia, the fruit of *R. apetalus* is used as a wild edible [23]. Hamill et al. [24] revealed the potential of crude methanolic extract and petroleum ether and chloroform fractions of *R. apetalus* to inhibit Gram positive and Gram negative bacteria and *Candida albicans*. The study carried out by Seshathri [21] showed the inhibitory potential of chewing stick of *R. apetalus* against *Candida albicans*. With Cinnamon, the chewing stick of *R. apetalus* was found to exhibit synergistic activity against *C. albicans*. The objective of present study was to detect phytochemicals and to evaluate antioxidant activity of leaves of *R. apetalus*. The objective of the present study was to estimate the content of total phenolics and flavonoid and to evaluate antioxidant activity of leaf extract of *R. apetalus*.

Materials and methods

Collection and identification of plant

The plant *R. apetalus* was collected at Nekemte, located in the East Welega Zone of the Oromia Region, Ethiopia. The plant was authenticated based on its characteristics by referring standard flora and other literatures [9,13].

Extraction

Maceration process was employed for extraction of powdered leaf material. The leaf powder (10g) was soaked in double distilled

water (100ml) in a stoppered container and the container was left for 3 days at room temperature. The container was stirred frequently. After 3 days, the contents were filtered and the filtrate was subjected to evaporation (in a rotary evaporator) to get concentrated extract [25].

Phytochemical analysis of leaf extract

The leaf extract was subjected to preliminary phytochemical analysis to detect the presence of constituents viz. glycosides (Keller-Killiani test), saponins (Foam test), flavonoids (Alkaline reagent test), alkaloids (Mayer's test, Dragendrodroff's test), phenolics and tannins (Ferric chloride test), phytosterols and triterpenoids (Leiberman-Bucharat test, Salkowaski test) and fixed oils and fats by standard phytochemical procedures [26,27,28].

Preparation of extract for antioxidant activity

The leaf extract was dissolved in methanol and diluted serially to obtain various concentrations viz. 6.25-200 µg/ml.

Antioxidant activity of leaf extract

Four in vitro assays viz. DPPH radical scavenging, ABTS radical scavenging, hydroxyl radical scavenging and lipid peroxidation inhibition assay, were carried out to investigate antioxidant potential of leaf extract of *R. apetalus*. Content of total phenolics and flavonoids were also estimated.

DPPH radical scavenging assay

The DPPH radical scavenging potential of various concentrations of leaf extract and ascorbic acid (reference standard) was investigated by following the protocol of Chandrashekar et al. [3]. Absorbance was measured at 517nm. DPPH radical scavenging activity (%) was calculated using the formula:

Scavenging of DPPH radicals (%) = 1-(absorbance of sample/absorbance of blank) x 100. IC_{50} value was calculated. IC_{50} value indicates the concentration of leaf extract/ascorbic acid required to scavenge 50% of free radicals.

ABTS radical scavenging assay

The scavenging of ABTS radicals by various concentrations of leaf extract and ascorbic acid (reference standard) was determined by employing the protocol of Jiang et al. [29]. Absorbance of reaction mixture was measured at 734nm. The scavenging of ABTS radicals (%) was calculated using the formula:

Scavenging of ABTS radicals (%) = 1-(absorbance of sample/absorbance of blank) x 100. IC_{50} value was calculated. IC_{50} value indicates the concentration of leaf extract/ascorbic acid required to scavenge 50% of free radicals.

Hydroxyl radical scavenging assay

The scavenging of hydroxyl radicals by various concentrations of leaf extract and ascorbic acid (reference standard) was evaluated by following the protocol of Jiang et al. [29]. Absorbance of reaction mixtures was read at 510nm. The scavenging of hydroxyl radicals (%) was calculated using the formula:

Scavenging of hydroxyl radicals (%) = 1-(absorbance of sample/absorbance of blank) x 100. IC_{50} value was calculated. IC_{50} value indicates the concentration of leaf extract/ascorbic acid required to scavenge 50% of free radicals.

Lipid peroxidation inhibition assay

The extent of inhibition of lipid peroxidation was evaluated by estimating the thiobarbituric acid reactive substances (TBARS) by employing the protocol of Badmus et al. [4] with minor modifications. Absorbance of reaction mixture was measured at 532nm. Ascorbic acid was used as reference standard. The percentage of inhibition of lipid peroxidation was calculated using the formula:

Inhibition of lipid peroxidation (%) = (Ac – At / Ac) x 100, where 'Ac' and 'At' refers to absorbance of control and absorbance of test respectively.

Total phenolic content

The content of total phenolics in the leaf extract of *R. apetalus* was estimated by Folin-Ciocalteu's reagent method as described by Raghavendra et al. [30]. Absorbance was measured at 750nm. Gallic acid was used as reference standard. The phenolic content was expressed as mg gallic acid equivalents/100g of dry extract.

Total flavonoid content

The content of total flavonoids in the leaf extract of *R. apetalus* was estimated by following the aluminium chloride colorimetric estimation method as described by Raghavendra et al. [30]. Absorbance was measured at 510nm. Catechin was used as reference standard. The flavonoid content was expressed as mg catechin equivalents/100g of dry extract.

Statistical analysis

The experiments were conducted in triplicates. The results are represented as Mean±Standard deviation (S.D). IC_{50} values were calculated by linear regression analysis using Origin (Data Analysis and Graphing) Software version 7.0 for windows.

Results and discussion

Phytochemicals detected in leaf extract of *R. apetalus*

Chemical compounds present in plants are called phytochemicals. Most of the phytochemicals (secondary metabolites) are unique to species or genera and are significant with respect to a range of bioactivities they display. Alkaloids, polyphenolic compounds (including flavonoids) and triterpenoids are few among the bioactive compounds present in the plants. The medicinal and therapeutic potential of plants is due to their phytochemical composition. Purified phytochemicals are shown to exhibit a myriad of biological activities such as antimicrobial, antioxidant, antidiabetic and anticancer activities. It becomes important to screen the plants or their extracts for the presence of various phytoconstituents [26,31-34]. The leaf extract of *R. apetalus* was screened for the presence of phytoconstituents by standard phytochemical procedures. In the extract, all phytochemicals except glycosides and fixed oils and fats were detected (Table 1).

DPPH radical scavenging activity of leaf extract of *R. apetalus*

The assay involving scavenging of DPPH radicals is one of the most widely used in vitro radical scavenging assays being used to evaluate the antiradical activity of various kinds of samples including plant extracts. DPPH is a stable, commercially available, ready to use, nitrogen centred, purple colored, organic free radical having an absorption maximum at 515-520nm in alcoholic solution. Substances having hydrogen donating potential convert the DPPH radical into non-radical form DPPHH which is evidenced by a change in the color from purple to yellow [3-6,29,35,36]. In the present study, we evaluated antiradical activity of leaf extract of *R. apetalus* by DPPH assay and the result obtained is presented in Figure 2. Both leaf extract and ascorbic acid scavenged DPPH radicals dose dependently. At 200µg/ml concentration, leaf extract and ascorbic acid exhibited a scavenging activity of 90.16% and 95.41% respectively. Ascorbic acid was found to exhibit marked scavenging of DPPH radicals (IC_{50} value 15.21µg/ml) when compared to leaf extract (IC_{50} value 22.49µg/ml). In an earlier study, methanol, 80% methanol and acetone extract of *R. apetalus* leaf were screened for scavenging of DPPH radicals. Methanol extract was most effective in scavenging radicals followed by acetone and 80% methanol extracts as indicated by IC_{50} values [1].

ABTS radical scavenging activity of leaf extract of *R. apetalus*

Like DPPH assay, the assay involving scavenging of ABTS radicals is another widely used in vitro assay for evaluating antiradical activity of several kinds of samples including plant extracts. However, the assay needs the generation of radicals which can be done by reacting ABTS stock solution with a strong oxidizing agent such as potassium persulfate. The reduction of blue-green colored ABTS radicals to neutral form (colorless) by antioxidants can be easily measured by the suppression of its characteristic long wave absorption spectrum [37-41]. In the present study, the leaf extract of *R. apetalus* was shown to exhibit dose dependent scavenging of ABTS radicals (Figure 3). A scavenging activity of >50% was observed at concentration of 12.5µg/ml and 25µg/ml of ascorbic acid and leaf extract respectively. Ascorbic acid scavenged ABTS radicals more efficiently (IC_{50} value of 4.78µg/ml) when compared to leaf extract (IC_{50} value of 17.53µg/ml). The leaf extract was more efficient in scavenging ABTS radicals when compared to DPPH radicals.

Hydroxyl radical scavenging activity of leaf extractof *R. apetalus*

Hydroxyl radical is a highly reactive free radical. It is the neutral form of hydroxide ion and it can strongly react with organic as well as inorganic molecules including DNA, lipids, proteins, and carbohydrates. Hydroxyl radical can cause severe damage to the cells than any other reactive oxygen species can do. Often, hydroxyl radicals are considered to be rapid initiators of lipid peroxidation. Hydroxyl radicals can be produced by Fenton reaction, in which H_2O_2 react with metal ions. Scavenging of hydroxyl radicals is considered as a very important antioxidant activity as hydroxyl

radicals are more damaging to biomolecules. Itis shown that extracts from plants display scavenging activity against hydroxyl radicals [4,7,29,35,37,42]. The result of scavenging of hydroxyl radicals by leaf extract of *R. apetalus* is shown in Figure 4. The leaf extract scavenged hydroxyl radicals dose dependently with an IC_{50} value of 33.43µg/ml. A scavenging activity of >50% by leaf extract was observed at 50µg/ml concentration. Ascorbic acid scavenged hydroxyl radicals more efficiently (IC_{50} value 25.37µg/ml) than leaf extract.

Lipid peroxidation inhibition by leaf extract of *R. apetalus*

The process of lipid peroxidation is known to occur via a free radical chain reaction. The process of lipid peroxidation results in damage to biological membrane leading to loss of membrane functioning (such as decreased fluidity of membrane, inactivation of membrane bound enzymes and receptors). The interaction of free radicals with the lipids results in the formation of lipid radicals. On reacting with molecular oxygen, these radicals form lipid peroxyl radicals which can further propagate the peroxidation process by abstracting hydrogen atoms from other lipid molecules. Substances that have the potential to donate hydrogen can break the propagation process and inhibit lipid peroxidation. Studies have shown that many plants possess the ability to inhibit lipid peroxidation through hydrogen donation [4,7,35,43,44]. The result of inhibition of lipid peroxidation by leaf extract of *R. apetalus* is shown in Figure 5. Both leaf extract and ascorbic acid displayed concentration dependent inhibition of lipid peroxidation. The extent of inhibition of lipid peroxidation was higher in case of ascorbic acid (IC_{50} value 23.577µg/ml) when compared to leaf extract (IC_{50} value 131.63µg/ml).

Total phenolic and flavonoid content of leaf extract of *R. apetalus*

Plants contain several classes of bioactive metabolites. Among these, polyphenolic compounds including flavonoids are considered to be most important plant constituents as they exert many biological activities. Recently, polyphenolic compounds received much attention due in part to their effective antioxidant activity. These aromatic secondary metabolites are distributed in various parts of the plant and are known to quench free radical and thereby lessen their deleterious effects. Consumption of plants with polyphenolic compounds seems to bring about health benefits especially through their antioxidant activity[8,30,33,39,45,46,47]. In the present study, the leaf extract of *R. apetalus* was estimated for total phenolic and flavonoid content by FCR method and aluminium chloride colorimetric estimation assay respectively. The phenolic and flavonoid content of aqueous extract of *R. apetalus* leaf was found to be 146.21mg gallic acid equivalents/100g of dry extract and 72.82mg catechin equivalents/100g of dry extract respectively.

Conclusions

The present study revealed potential in vitro antioxidant activity of leaf extract of *R. apetalus*. The extract was effective in scavenging DPPH, ABTS and hydroxyl radicals and inhibiting lipid peroxidation. The observed antioxidant activity could be ascribed to the presence of phenolic compounds and flavonoids. The leaf extract was shown to exhibit lesser activity when compared to reference standard, however, it is evident from the result of the present study that the plant possesses radical scavenging potential which can be beneficial with respect to inhibition of lipid peroxidation. In suitable form the plant can be exploited to manage oxidative damage induced by free radical generation.

Sources of support

None

Conflicts of interest

None declared

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Lipid peroxidation inhibition and antiradical activities of some leaf fractions of *Mangiferaindica*. Acta Pol Pharm 2011; 68(1): 23-29.
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Table 1: Phytochemicals detected in leaf extract of *R. apetalus*

Phytochemicals	*R. apetalus* leaf extract
Saponins	+
Alkaloids	+
Flavonoids	+
Phenolics & tannins	+
Phytosterols & triterpenoids	+
Glycosides	-
Fixed oils and fats	-

'+' Detected; '-' Not detected

Figure 1: *Rubus apetalus*Poir. (Photograph by Raghavendra H.L)

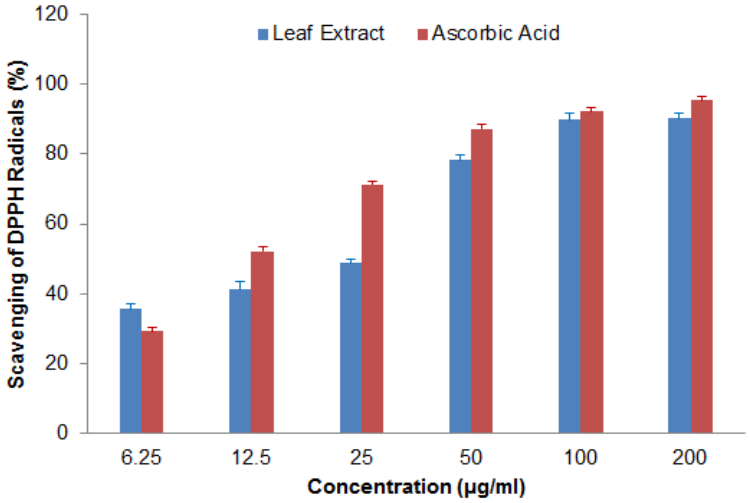

Figure 2: Scavenging of DPPH radicals by leaf extract and ascorbic acid

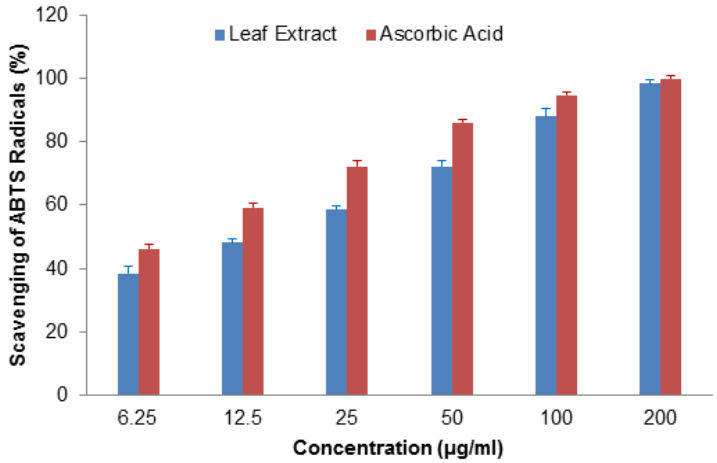

Figure 3: Scavenging of ABTS radicals by leaf extract and ascorbic acid

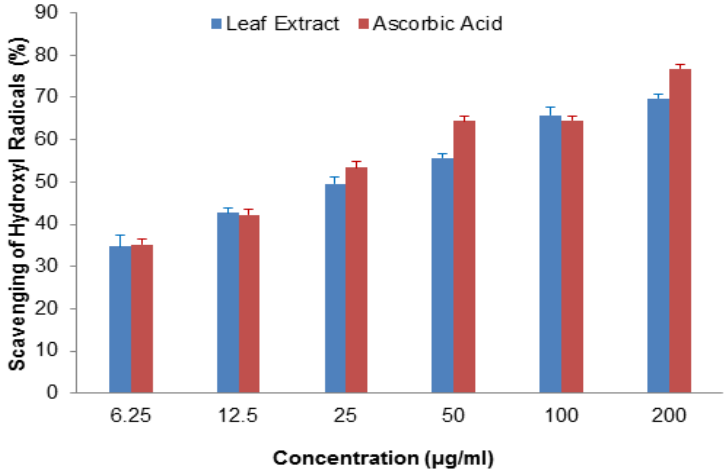

Figure 4: Scavenging of hydroxyl radicals by leaf extract and ascorbic acid

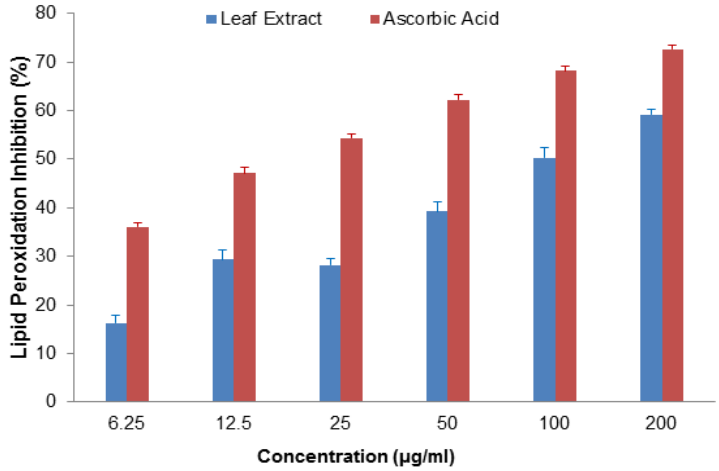

Figure 5: Inhibition of lipid peroxidation by leaf extract and ascorbic acid