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ANTIOXIDANT PROFILE OF *HAEMATOCARPUS VALIDUS* (MIERS) BAKH.F. EX FORMAN (KHOONPHAL) LEAF AND FRUIT : A MEDICINALLY IMPORTANT RARE ETHNIC FRUIT CROP

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Abstract

The present study was undertaken for the antioxidant profiling of the lesser known underexplored ethnic fruit crop *Haematocarpus validus* (Miers) Bakh.f. ex Forman (Khoon phal). Reports on nutritional status and phytoconstituents of this plant are scanty and there is no scientific report on antioxidant potential of *H. validus* leaf eventhough, it is used as a hepatoprotective and anti-inflammatory agent in ethnomedicine. Methanolic extracts prepared from shade dried and powdered samples of both leaf and fruit were used in the study. The secondary metabolite content recorded were: total phenolics - 113.33 and 86.67 mg GAE/g, flavonoids - 67.93 and 59.63 mg QE/g and alkaloids - 2.72 and 1.93% for leaf and fruit, respectively. *H. validus* methanolic leaf and fruit extracts are found to be a potent source of natural antioxidants as it was evidenced by DPPH free radical scavenging activity and DNA protective properties (at 500 µg/ml concentration) *in vitro*.

Key words : DPPH assay, flavonoids, *H. validus,* Khoon phal, phenolic compounds.

Introduction

Haematocarpus validus (Miers) Bakh.f. ex Forman (Blood fruit - Khoon phal) is a lesser known large woody perennial climber belonging to the family Menispermaceae. The name *Haematocarpus* (Greek-Heima-blood; karpos- fruit) refers to the red colour of fruits (blood-red fruits). The plant is distributed in isolated pockets of tropical Asia (Thailand, Bangladesh, Indonesia, India: Assam, Tripura, Meghalaya, Mizoram, Andaman and Nicobar Islands), Sumatra and Java. This edible fruit crop is popular among the tribal populations of the hot and humid tropics of Asia and also reported as a folklore medicine for various ailments including jaundice, inflammatory conditions, anaemia etc. (Singh *et al*., 2014, Rahim *et al*., 2015, Bohra *et al*., 2016; Momin *et al*., 2016; Alex *et al*., 2017; 2018).

Active oxygen species (AOS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, singlet oxygen, *etc.* are free radicals and must be present in the cell as they act as signaling molecules (Halliwell and Gutteridge, 1999). But these molecules should be present at very low levels and excess production can result in oxidative stress and thus causes damage to cell organelles and DNA, introduce gene mutation and ultimately various debilitating diseases including neurodegenerative and cardiovascular diseases, aging, cancers, etc. (Nilsson *et al*., 2004; Shen *et al*., 2010). Flavonoids and phenolic compounds of plants have been reported to exert multiple biological effects including antioxidant and free radical scavenging abilities (Sangameswaran *et al*., 2009; Sreelatha and Padma, 2009). Antioxidant-based drugs and formulations from plants for the prevention and treatment of oxidative stress related diseases have attracted a great deal of research interest in natural antioxidants (Nagavani *et al*., 2010). Considering the safety aspects and side effects of synthetic antioxidants, natural antioxidants are projected as green alternatives (Sudha and Srinivasan, 2014; Pirzadah *et al*., 2017).

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Materials and Methods

Antioxidant property was studied with methanolic extract prepared from leaf and fruit (without seed) samples of *H. validus*. The shade dried and powdered material was first defatted with petroleum ether (60-80

^{0}C) and then extracted with methanol using soxhlet apparatus. The solvent was recovered from the extract at reduced pressure using a rotary evaporator.

Analysis for total phenolic compounds

The total phenolic compounds (TPC) were analyzed using the Folin-Ciocalteau method with some modification (Ghafoor and Choi, 2009). To estimate TPC, 200 µl of methanolic extract (1 mg/ml) was made up to 3 ml with distilled water and mixed with 500 µl Folin-Ciocalteau reagent. After incubation for 10 min at RT, 2 ml of 20 % Na_2CO_3 solution was added, mixed immediately and incubated for 2 hrs in the dark. The absorbance was read at 765 nm. Gallic acid of 1 mg/ml was prepared as the standard and various concentrations (20-100 µg) of this used for the preparation of calibration curve. TPC was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g d.wt.) of the extract.

Estimation of total flavonoid content: The total flavonoid content (TFC) of *H. validus* leaf and fruit extracts was determined by aluminium chloride colorimetric method (Chang *et al.*, 2002). Ten milligram of quercetin was dissolved in 100 ml of 80 % methanol (100 µg/ml) and various concentrations (20 - 100 µg) of this were used for calibration curve. The required volume of standard solutions were mixed with 1.5 ml of methanol (95%), 0.1 ml of 10 % $AlCl_3$, 0.1 ml of 1 M potassium acetate and made up to 5 ml with distilled water. After incubation at RT for 30 min, absorbance of the mixture was measured at 415 nm. The amount of 10% $AlCl_3$ was substituted with distilled water in blank. 100 mg of methanolic extract was dissolved in 2.5 ml of 80% methanol (40 mg/ml). Further, 0.5 ml of extracts was reacted with $AlCl_3$ as described above. TFC was expressed as mg quercetin equivalent per g dry weight (mg QE/g d.w.) of the extract.

Estimation of total alkaloid content

Alkaloids were determined by gravimetric method of Harborne (2005). Powdered leaf and fruit samples (5 g each) were added separately to 10% NH_4OH, stirred and allowed to stand for 4 hrs and filtered. The filtrate was evaporated to 1/4th of the original volume on a hot plate. To this, concentrated NH_4OH was added drop wise and the precipitate was filtered using a pre-weighed filter paper and washed with 10% NH_4OH solution. It was kept in an oven for 30 min at 60^{0}C and reweighed.

The amount of alkaloids present was calculated as:

$$\% \text{ alkaloid} = \frac{W_2 - W_1}{W} \times 100$$

Where, W_1: wt. of the filter paper; W_2: wt. of filter paper with alkaloids; W: wt. of sample taken.

DNA protection assay

DNA protection capability of both leaf and fruit extracts was performed by the method of Lee *et al.* (2002). For this 5µl of plant extract (500 µg/ml) and 5 µl of pUC18 (0.5 mg/ml 10mM Tris HCl with 1 mM EDTA, pH 8.0) plasmid DNA (Sigma Aldrich) were mixed, vortexed and incubated for 10 min at RT. To this mixture, 5µl of Fenton's reagent (50 µM ascorbic acid, 30 mM H_2O_2 and 80 µM $FeCl_3$) was added and incubated for 10 min at 37 ^{0}C. The DNA was analyzed using agarose gel electrophoresis and observed under UV transilluminator.

DPPH radical scavenging activity

The radical scavenging activity of methanolic extracts of leaf and fruit samples was determined by using DPPH assay (Blois, 1958). The decrease in the absorbance of DPPH solution after the addition of plant extract was measured at 517 nm. Ascorbic acid (10 mg/ml methanol) was used as reference. Different volumes of extracts 1.25 µl - 20 µl (12.5 - 200 µg) from a stock of 10 mg/ml were made up to a final volume of 20 µl with methanol and 1.98 ml freshly prepared DPPH (0.1 mM). The components were mixed properly. A control without the test compound, but an equivalent amount of distilled water was taken. After 30 min incubation in dark at RT, the absorbance of the reaction mixture was read at 517 nm. The radical scavenging activity was expressed as % inhibition of DPPH using the formula:

$$\% \text{ inhibition} = \frac{\text{Control-test}}{\text{Control}} \times 100$$

Results and Discussion

Total phenol and flavonoid content

TPC of the methanolic extracts prepared from the leaves and fruits of *H. validus* were estimated by using the calibration curve ($Y = 0.010X + 0.0105$; $R^2 = 0.9959$) prepared with gallic acid as standard (fig. 1). TFC of the samples were determined using quercetin as the standard ($Y = 0.0096X + 0.0053$; $R^2 = 0.9882$) (fig. 2). TPC and TFC of leaf extract was found to be 113.33 ± 4.41 mg GAE/g and 67.93 ± 2.01 mg QE/g, respectively, which was moderately higher when compared to fruit extract (TPC 86.67 ± 2.88 mg GAE/g and TFC 59.63 ± 1.49 mg QE/g) (table 1).

Total alkaloid content

Total alkaloid content of the leaf and fruit samples of *H. validus* was found to be 2.72 ± 0.08% and 1.93 ± 0.06%, respectively. Based on this estimation, it can be

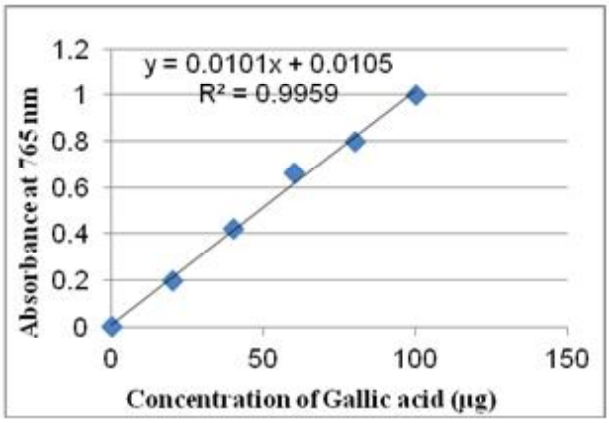


Fig. 1 : Calibration curve for TPC.

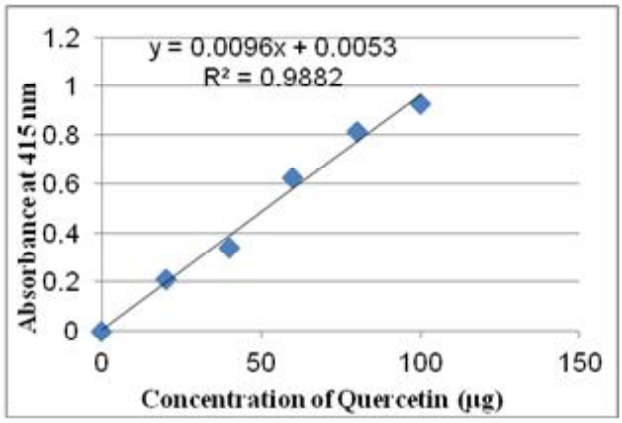
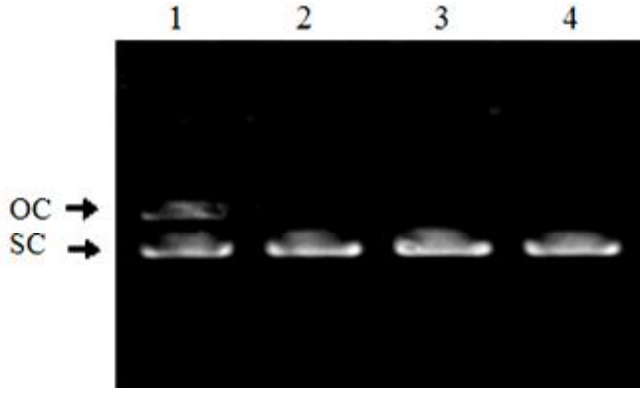


Fig. 2 : Calibration curve for TFC.



Lane 1: Fenton's reagent, 2: Fenton's + Leaf extract, 3: Fenton's + Fruit extract, 4: Control
OC - Open circular; SC – Super Coiled

Fig. 3 : DNA protection assay.

put forward that, *H. validus* is rich in alkaloids just as most of the other plants belonging to the family Menispermaceae.

DNA protection assay

Antioxidant activity of the plant extracts was evaluated in terms of their ability to quench hydroxyl radical. The methanolic leaf and fruit extract of *H. validus* at 500 µg/ml concentration exhibited remarkable protection against the DNA damage induced by hydroxyl radicals which are generated during Fenton's reaction as it was evident from the band pattern obtained for control and test reaction. The DNA protection assay carried out with leaf and fruit extracts clearly indicated the protective efficacy of these extracts as there was no nicking of supercoiled (SC) form of pUC18 plasmid used in the study (fig. 3) and it was also comparable to the normal control (lane 4). In the negative control (lane 1) where the plasmid DNA was mixed up with Fenton's reagent alone, nicking took place and resulted in the open circular (OC) form of the molecule.

Table 1 : Total phenolic and flavonoid content in leaf and fruit extract of *H. validus*.

Plant samples	TPC (mg GAE/g)	TFC (mg QE/g)
Leaf	113.33 ± 4.41	67.93 ± 2.01
Fruit	86.67 ± 2.88	59.63 ± 1.49

Values are expressed as mean ± SE

DPPH radical scavenging activity

DPPH radical scavenging activity of *H. validus* extracts (both leaf and fruit) was tested and compared with the standard antioxidant ascorbic acid. Both the extracts were found to have comparable free radical scavenging activity with the standard compound and this was also highly concentration dependent. There was a reciprocal relationship between the dose and scavenging property. Leaf extract had shown highest scavenging activity of 77.88 ± 1.29% at the concentration 200 µg/ml, while fruit extract showed 72.21 ± 0.86% at this concentration. The positive control, ascorbic acid recorded the maximum radical scavenging activity of 86.54 ± 0.51% at 200 µg/ml concentration (fig. 4).

Phenolic compounds have redox properties, which allow them to act as antioxidants (Soobrattee *et al*., 2005). As their free radical scavenging ability is facilitated by their hydroxyl groups, the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. As antioxidants, ûavonoids have been reported to be able to interfere with the activities of enzymes involved in reactive oxygen species generation, quenching free radicals, chelating transition metals and rendering them redox inactive in the Fenton reaction (Heim *et al.*, 2002).

Folin-Ciocalteu reagent assay is commonly used to determine the total phenolic content (TPC) of plant extracts. Ishiwata *et al*. (2004) reported TPC of dried fruits as mg of ascorbic acid equivalents (AAE)/100g

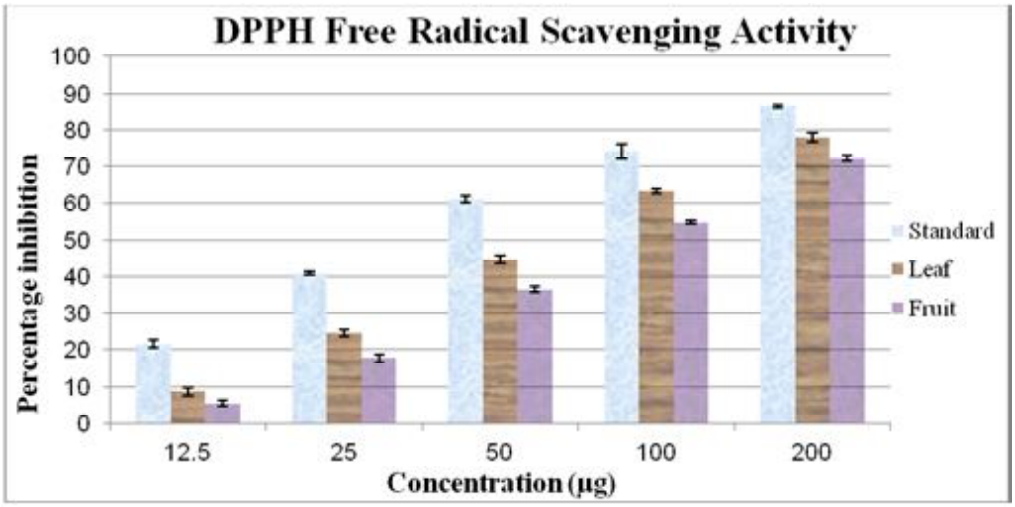


Fig. 4 : DPPH free radical scavenging activity.

and it was in the range of 916-2414 and the highest content was recorded for raisins followed by apricots, cranberries, peaches, figs, pears and prunes. Another study performed by Wu *et al*. (2004) expressed TPC as mg gallic acid equivalents per gram (mg GAE/g) of dried fruits and it decreased in the order prunes > raisins > figs > dates. However, in another study, Vinson *et al*. (2005) demonstrated highest TPC content (as catechin equivalents) for dates (1959 mg CE/100g f.wt.) and figs had the least amount (320 mg CE/100g f.wt.) among six dried fruits- apricots, cranberries, dates, figs, plums and raisins. The total phenolic contents in extracts obtained from the stems and leaves of coriander, mint and parsley were studied by Al-Juhaimi and Ghafoor (2011). The highest contents (1.24 mg GAE/100ml) were observed in the extract of mint (*Mentha arvensis*) leaves followed by parsley (*Petroselinum crispum*) leaves (1.22 mg GAE/100ml) and coriander (*Coriandrum sativum*) leaves (1.12 mg GAE/100ml). TPC of two Turkish fig varieties were reported to be 193 and 417 mg GAE/100g d.wt. (Kamiloglu and Capanoglu, 2015). The results and inferences from different studies may differ substantially as different methods are adopted and different standard compounds have been used for the analyses.

In 1963, Hegnauer opined that those plants which contain >0.01% alkaloid can be considered as alkaloid rich. Different classes of alkaloids especially isoquinoline, aporphine, protoberberine, *etc*. were previously reported from Menispermaceae by several researchers (Nakaoji *et al*., 1997; Camacho, 2000; Goren *et al*., 2003; Chen *et al*., 2005; Otshudi *et al*., 2005; Zhang and Yue, 2005). Recently, Alex *et al*. (2017) reported the presence of different alkaloids in leaf and fruit extracts of *H. validus* such as sinomenine (leaf), ecgonine (leaf), reticuline (fruit), ambelline (fruit), metanephrine (fruit) and choline (leaf and fruit).

Hydrogen peroxide and ferric chloride induce DNA damage through oxidative stress, which is attributed to the production of hydroxyl radicals, and these radicals act on supercoiled DNA causing its break down into open circular and sometimes linear form (Naqvi *et al*., 2010; Ali *et al*., 2015). Phenolic compounds are reported to provide superior free radical scavenging property to fruits, nuts and cereals (Carlsen *et al*., 2010). DPPH (1,1-Diphenyl-2-picrylhydrazyl), a relatively stable free radical is found to be effectively scavenged by different types of phenolic compounds and flavonoids as they can act as H^+ donors. When DPPH acquire proton the purple colour of the solution turns yellow. In general, the total phenolics, flavonoids and free radical scavenging activity of *H. validus* leaf extract were slightly higher than fruit extract.

According to the present study, there was a positive correlation between the antioxidant activities and total phenolics of the plant extracts. Many studies articulated the positive correlation between high phenolic and flavonoid content and strong antioxidant activities (Cimpoiu *et al*., 2007; Ghafoor and Choi, 2009; Lim *et al*., 2010; Razab and Abdul Aziz, 2010; Alex *et al*., 2013) of plants. It is a widely accepted fact that plants rich in phenolic and flavonoid content to have strong free radical scavenging potential (Bursal *et al*., 2013).

In conclusion, the ethnomedicinal uses of *H. validus* are well supported by the present findings which show both leaf and fruit extracts are rich in the content of phenolics, flavonoids and alkaloids. In addition these extracts are having good free-radical scavenging and DNA protective properties. Therefore, this rare ethnic fruit crop can be promoted for its extensive cultivation

and further its use in nutraceutical and pharmaceutical preparations.

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