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Free Radical Scavenging Activity and Chemical Constituents of the Unripe Fruits of *Spondias pinnata* (L.f.) Kurz. from Nepal

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Abstract

Spondias pinnata (L.f.) Kurz.(Anacardiaceae) is widely used as food and for medicinal properties. This study aims to disclose the free radical scavenging potential, total phenolic and flavonoid contents and phytochemical constituents of 70% methanol extract of unripe fruits of *S. pinnata* collected from Kaski district, Nepal. The free radical scavenging activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay method. The total phenolic content (TPC) and total flavonoid content (TFC) were estimated by using Folin-Ciocalteu's phenol reagent and aluminium chloride methods, respectively. *S. pinnata* fruits extract showed potent free radical scavenging activity with IC_{50} value 2.75±0.23 µg/ml. TPC and TFC values were found to be 229.24±0.46 mg GAE/g and 192.58±3.81 mg QE/g, respectively. Detailed chemical isolation of the extract afforded caffeic acid methyl ester (**1**) and rhamnetin 3-*O*-sophoroside (**2**). In conclusion, *S. pinnata* fruits were found to be rich source of phenolic and flavonoid compounds and possessed strong free radical scavenging property. However, further study is needed to explore its potential health benefits and bioassay guided chemical analysis should be performed to isolate and identify the bioactive compounds.

Key Words: *Spondias pinnata*, DPPH, Total phenolic content, Total flavonoid content, Isolation

1. Introduction

Medicinal plants, both in crude and refined form, are considered as an important therapeutic aid for treatment of diseases in humans and animals. More than 70% of world's population depend on traditional medicinal plants to maintain their health and cure their illness (Joseph and Jini, 2013). The chemical constituents obtained from different parts of medicinal plants serve as lead molecules in the development of modern medicines and also as potent components of various functional foods and nutraceuticals (Atanasov et al., 2015; Ayaz et al., 2019; Yeung et al., 2019). Therefore, medicinal plants need to be investigated for biological activities and phytochemical composition to provide safe and effective remedies.

Spondias pinnata (L. f.) Kurz. (Synonyms: *Mangifera pinnata* L. f., *Spondias acuminata* Roxb., *Spondias mangifera* Willd.) (Figure 1) is a deciduous tree belonging to family Anacardiaceae. It is mostly found in lowlands and hill forests of Nepal, Bhutan, China (southern), India and Myanmar. In Nepal, it is locally known as "Amara" and fruits are eaten fresh or pickled (Manandhar, 2002) and bark juice is given for stomach ache, diarrhea, dysentery and rheumatism (Bora et al., 2014). *S. pinnata* leaves are used in the formulation of an herbal beverage which is consumed to treat heart burn, urolithiasis and diabetes and to improve immunity in Indonesia (Sujarwo and Keim, 2019). Previous studies have reported the hypoglycemic activity of methanol extract (Dash and Mondal, 2009), anti-hyperlipidaemic and insulinotropic effects of aqueous extract (Attanayake et al., 2014) and cytotoxic activity of the methanol extract (Ghate et al., 2014) of the bark. Likewise, antioxidant activities of methanol extract of bark (Hazra et al., 2008) and leaves (Sai et al., 2019) are also reported.

In addition, the phytochemical and nutritional characterization of raw fruits has demonstrated various constituents such as phenolic compounds, flavonoids, amino acids and minerals (Satpathy et al., 2011) and triterpenoids such as β-amyrin and oleanolic acid (Sameh et al., 2018) . Recently, we reported the total phenolic content (TPC), total flavonoid content (TFC), antioxidative and α-amylase inhibitory activity of the methanol extract of the leaves of *S. pinnata* (Sai et al., 2019). In continuation, this this paper, we aimed for the evaluation of the free radical scavenging activity, estimation of TPC and TFC and isolation of secondary metabolites from the unripe fruits of *S. pinnata*.

Figure 1. The unripe fruits of *S. pinnata* (a) and dried peels and pulp (b) used for experiments

2. Materials and Methods

2.1. Instruments and chemicals

^{1}H- and ^{13}C-NMR spectra were measured on BRUKER AVANCE 600 NMR Spectrometer (Bruker, Billerica, MA, USA). Column chromatography (CC) was carried out with MCI gel CHP20P (75~150 μm, Mitsubishi Chemical Industries Co. Ltd., Tokyo, Japan), Sephadex LH-20 (Amersham Pharmacia Biotech, Tokyo, Japan), and silica gel 60 (0.040–0.063 mm, Merck KGaA, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on a pre-coated silica gel 60

F254 (Aluminum sheet, Merck KGaA, Darmstadt, Germany). DPPH, gallic acid and quercetin were purchased from Wako Pure Chemicals, (Tokyo, Japan). Ascorbic acid and aluminium chloride were procured from Qualigens Fine Chemicals, India. Folin-Ciocalteu's phenol reagent was from Sigma Aldrich, USA.

2.2. Plant collection and extraction

The unripe fruits of *S. pinnata* were collected from Kaski district, Gandaki province, Nepal in the month of August, 2017 and were identified by Dr. Radheshyam Kayastha, former Professor, Tribhuvan University, Nepal. A voucher specimen of this plant (PUCD-2018-07/08) was deposited at the Laboratory of Pharmacognosy, Pokhara University, Nepal. The fruits were thoroughly washed with tap water and peeled along with pulp to remove seeds. Air dried peel and pulp (1 kg) of *S. pinnata* was extracted twice with 70% methanol at room temperature by maceration. The filtered extract was evaporated using rotary evaporator to obtain 240 g of dried extract. The dried extract was stored in refrigerator and used for further experiments.

2.3. Evaluation of DPPH free radical scavenging activity and TPC and TFC

Evaluation of DPPH free radical scavenging activity and estimation of TPC and TFC values were performed according to the method described in previous paper (Sai et al., 2019).

2.4. Chemical isolation

S. pinnata fruits extract (236.0 g) was suspended in water and applied to MCI gel CHP-20P column. The column was then eluted with water, by 40% methanol, 70% methanol and methanol to obtain 45 fractions each of 100 mL. TLC pattern of each fractions were observed in suitable solvent system and fractions with similar spots were combined to obtain 10 major fractions (SPFW-1 to SPFW-10).

Fraction SPFW-8 (1.23 g) was then applied into Sephadex LH-20 column and eluted with 50% methanol followed by methanol to obtain 6 sub-fractions (SPFW-8-1 to SPFW-8-5). SPFW-8-5 was obtained as pure compound **1** (100 mg). Subfraction SPFW-8-2 (180 mg) was further purified by silica gel column chromatography and eluted with $CHCl_3$: MeOH: H_2O (8:2:0.2) to obtain compound **2** (70 mg).

2.5. Caffeic acid methyl ester (1)

White amorphous power. ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 7.53 (1H, d, J = 15.9 Hz, H-7), 7.03 (1H, d. J = 2.0 Hz, H-2), 6.93 (1H, dd, J = 2.0 Hz, 8.2 Hz, H-6), 6.77 (1H, d, J = 8.2 Hz, H-5), 6.25 (1H, d, J = 15.9 Hz, H-8), 3.75 (3H, s, OCH_3). ^{13}C-NMR (150 MHz, CD_3OD)□□C: 166.9 (C-9), 148.3 (C-4), 145.5 (C-8), 145.1 (C-3), 129.3 (C-1), 121.3 (C-6), 115.7 (C-5), 114.7 (C-2), 111.3 (C-7), 51.1 (OCH_3) (Fujioka et al., 1999).

2.6. Rhamnetin 3-*O*-sophoroside (2)

Pale amorphous powder. ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 7.70 (1H, d. J = 2.1 Hz, H-2'), 7.57 (1H,dd. J = 2.1, 8.5 Hz, H-6'), 6.90 (1H, d. J = 8.5 Hz, H-5'), 6.58 (1H, d. J = 2.1 Hz, H-8), 6.33 (1H, d. J = 2.1 Hz, H-6), 5.37 (1H, d. J = 7.6 Hz, H-1"), 4.77 (1H, d. J = 7.6 Hz, H-1"'), 3.88 (3H, s, OCH_3), 3.20-3.80 (remaining sugar protons). ^{13}C-NMR (150 MHz, CD_3OD) δ_C :179.8 (C-4), 167.2 (C-7), 162.7 (C-5), 159.3 (C-2), 158.3 (C-9), 149.9 (C-4'), 145.9 (C-3'), 135.3 (C-3), 123.2 (C-6'), 122.8 (C-1'), 117.9 (C-2'), 116.2 (C-5'), 106.6 (C-10), 104.9 (C-1"'), 101.2 (C-1"), 99.1 (C-6), 93.1 (C-8), 82.8 (C-2"), 78.2 (C-5"), 78.1 (C-5"'), 77.9 (C-3", C-3"'), 75.6 (C-2"), 71.4 (C-4"'), 71.1 (C-4"), 62.4 (C-6", C-6"'), 56.6 (OCH_3) (Goda et al., 1999).

2.7. Statistical analysis

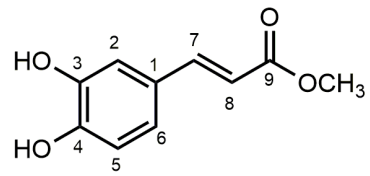
Results are expressed as mean ± SD (n=3). All the data analysis was carried out using Microsoft Excel 2007.

3. Results and Discussion

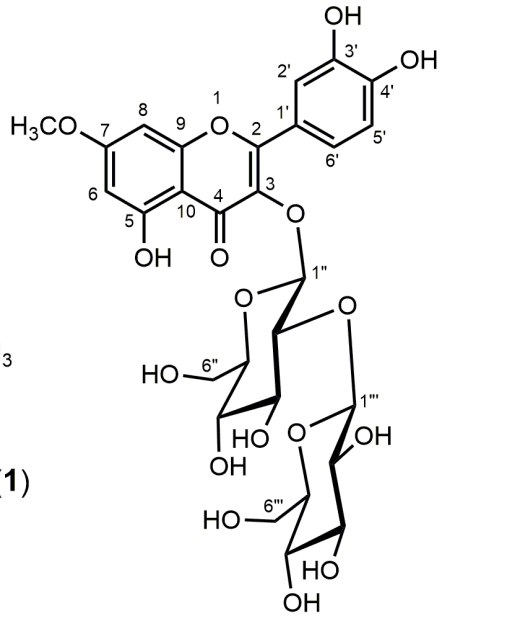
Various plants belonging to genus *Spondias* are used in traditional medicine systems and are studied for their chemical constituents and pharmacological activities (Sameh et al., 2018). In this study, we evaluated the free radical scavenging activity of the 70% methanol extract of dried peels and pulp of unripe fruits of *S. pinnata* collected from western Nepal. Additionally, estimation of TPC and TFC and isolation of secondary metabolites was also performed. Antioxidant activity of *S. pinnata* fruits extract was evaluated by using DPPH free radical scavenging assay method and expressed in terms of IC_{50} values (µg/ml) (Table 1). *S. pinnata* fruits extract showed strong antioxidant activity with IC_{50} value of 2.75 ± 0.23 µg/ml compared to positive control, ascorbic acid (IC_{50} = 3.16 ± 0.02 µg/ml). The total phenolic (TPC) and flavonoid content (TFC) values of *S. pinnata* fruits extract were found to be 229.24±0.46 mg gallic acid equivalent (GAE)/g and 192.58±3.81 mg quercetin equivalent (QE)/g, respectively (Table 1). The strong antioxidant activity of *S. pinnata* fruits can be attributed to the presence of phenolics and flavonoids which are already established as potent free radical scavengers by many studies (Satpathy et al., 2011; Dirar et al., 2019) and it is further supported by the results of total phenolic and flavonoid contents in this study.

Table 1. IC_{50} values for DPPH assay, and TPC and TFC values of extract of *S. pinnata* fruits

Sample	IC_{50} values for DPPH assay (µg/ml)	TPC (mg GAE/g)	TFC (mg QE/g)
S. pinnata extract	2.75 ± 0.23	229.24 ± 0.46	192.58 ± 3.81
Control(ascorbic acid)	3.16 ± 0.02	-	-



caffeic acid methyl ester (**1**)



rhamnetin 3-*O*-sophoroside (**2**)

Figure 2. Structures of isolated compounds

The detailed chemical analysis of the extract afforded two compounds. The structures of these compounds were elucidated on the basis of NMR spectroscopic data and comparison with literature values as caffeic acid methyl ester (**1**) (Fujioka et al., 1999). and rhamnetin 3-*O*-sophoroside (**2**) (Goda et al., 1999) (Figure 2.). Caffeic acid methyl ester (methyl caffeate) is a hydroxycinnamic acid derivative and rhamnetin 3-*O*-sophoroside (7-*O*-methylquercetin 3-*O*-sophoroside, **2**) is a flavonoid glycoside. Previous study on raw fruits of *S. pinnata* has reported the presence of other hydroxy cinnamic acid derivatives i.e. chlorogenic acid and *p*-coumaric acid and hydroxybenzoic acid derivatives i.e. gallic acid, salicylic acid and ellagic acid. This is the first report on isolation of caffeic acid methyl ester (**2**) from *S. pinnata.* However, it should be noted that it can be an artifact generated during isolation procedures. It has been also reported from various plant sources such as *Angelica japonica* A.Gray (Apiaceae) (Fujioka et al., 1999), *Heynea trijuga* Roxb. ex Sims (Meliaceae) (Devkota et al., 2014), *Phegopteris decursivepinnata* Fée (Thelypteridaceae) (Watanabe et al., 2018) among others. Rhamnetin 3-*O*-sophoroside (**2**) has been reported from only two sources previously i.e. *Nasturtium officinale* R.Br. (Brassicaceae) (Goda et al., 1999) and *Ranzania japonica* T. Ito (Berberidaceae) (Iwashina and Kitajima, 2009). Goda et al. (1991) also reported the strong inhibitory activity of compound **2** on histamine release from RBL-2H3 cells induced by antigen stimulation.

There are more than 8000 naturally occurring phenolic compounds including flavonoids reported from the medicinal plants, fruits and vegetables. Flavonoids are the largest group of polyphenolic compounds which are reported to have several pharmacological activities such as anti-inflammatory, antimicrobial, antidiabetic, antithrombogenic, hepatoprotective and antitumor activities (John et al., 2014; Khan et al., 2019). For example, quercetin exhibited anti-inflammatory activity by inhibiting cyclooxygenase and lipooxygenase pathways and thus reducing the formation of inflammatory mediators (Kim et al., 1998). Similarly, quercetin is reported to possess antidiabetic activity by regeneration of pancreatic cells in streptozotocin-induced diabetic rats (Vessal et al., 2003). Flavonoids and polyphenolic compounds are primarily known for their free radical scavenging activities. Their polyphenolic nature enables them to scavenge injurious free radicals such as superoxide anion and hydroxyl radicals (Adhikari-Devkota et al., 2018). The possible mechanism of antioxidant action can be suppressing the formation of reactive oxygen species by inhibition of enzymes or chelating trace elements (Pietta, 2000). Flavonoids have been found to inhibit enzymes such as xanthine oxidase and protein kinase C as well as chelate some trace elements which are responsible for reactive oxygen species generation (Ursini et al., 1994).

4. Conclusion

In conclusion, the unripe fruits of *S. pinnata* possessed strong free radical scavenging activity along with high content of total phenols and flavonoids. Two compounds, caffeic acid methyl ester (**1**) and rhamnetin 3-*O*-sophoroside (**2**) were isolated from 70% methanolic extract of the fruits. Further research should focus on the *in vivo* bioactivity evaluation of the extract and compounds and isolation and structure elucidation of other bioactive compounds.

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former Professor, Tribhuvan University, Nepal for plant specimen identification.

Conflicts of Interest

The authors declare no conflict of interest.

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