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Proximate Analysis and Comparative Evaluation of Antioxidant, Antidiabetic and Antibacterial Activities of *Capsicum Species* Consumed in Nepal

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Abstract

Chili has been consumed worldwide for a long time and is known for its aroma, hot taste, vibrant color, and its richness in various vitamins, minerals, capsaicin, and carotenoids, which offer protective attributes against health complaints such as rheumatism, joint pain, bronchitis, and cancer. This study aims to conduct a proximate analysis, phytochemical composition, estimation of total phenolic and flavonoid content, and antioxidant, antidiabetic, and antibacterial activities of three commonly used chili species in Nepal. Proximate analysis showed the presence of the highest percentage of crude fiber (16.43%), carbohydrate (13.59%), and fat (1.47%) in *Capsicum frutescence.* Using the Folin-Ciocalteu reagent method, the phenolic content was found in the range from 71.80 ± 3.36 mg GAE/g in *Capsicum frutescens* to 6.59 ± 0.50 mg GAE/g in *Capsicum annuum* var. *cerasiforme.* Similarly, the total flavonoid content was in the range of 22.38 ± 2.56 mg QE/g in *C. frutescens* to the minimum of 3.20 ± 0.43 mg QE/g in *C. annuum var. annuum.* The methanol extract of *Capsicum frutescens* was found to exhibit significant antioxidant capacity (IC_{50} = 99.13 ± 3.55 µg/mL). The extract exhibited moderate α-amylase inhibition capacity on evaluation by $CNPG_3$ method but did not show antibacterial activity against both gram negative and Gram-positive bacteria. The results of this study validate the traditional use of chili as nutraceutical and therapeutic purposes.

Keywords: *Red pepper, proximate analysis, DPPH, Folin-Ciocalteu reagent*

1. Introduction

Different cultivars of chili peppers are originated in Mexico and distributed all over the world. It is one of the oldest plant used in food and traditional medicine for the management of different

health complications (Saleh et al., 2018). Chili is valued for its sharp taste, aroma and used in several cousins for seasoning, in fresh and processed vegetables, spices, food coloring, and various cosmetics and therapeutic purposes (Paran & Knaap, 2007). It contains important minerals, vitamins A, B, C, and E and several phytochemicals including phenolics, carotenoids, and capsaicin. These bioactive compounds are responsible for the preventive therapeutic properties and are effective against many health problems such as cancer, rheumatism, bronchitis, joint issues, chest pain, common cold, headache, and coughs (Zaharan et al., 2022). The major active secondary metabolites abundant in the fruits and seeds of chili are capsaicin and allied molecules including dihydrocapsaicin, and capsicum carboxamide. Several studies have focused on the metabolism of these active compounds because of their unique properties, including antioxidant, anti-tumor, anti-inflammatory effects, as well as their role in weight regulation, diurnal-circadian regulation, and heart-related problems (Peng et al., 2023).

Chilies are small herbaceous plants belonging to the genus Capsicum in the Solanaceae family. They produce berry-like seeds that develop in the placenta, rather than being embedded in the fleshy pericarp. There are about 31 species of capsicum genus out of which only five are cultivated. Most of the chilies grow fruits of varying shapes and sizes and possess different taste, flavor and texture (Azlan et al., 2022). During ripening, chili fruits change to red, orange, and yellow colors due to the formation of carotenoid pigments. About 30 different pigments have been reported in red chili berries. The daily intake of chili with ascorbic acid, natural pigments, and several anti-oxidant compounds can minimize the risk of various disease including cardiovascular disease and cancer (Subedi et al., 2018).

The study of bioactive compounds in traditional foods plays a vital role in standardizing functional constituents in local plants. It also helps to understand the physiological effects of chili products, assisting in refining their quality. In this study, the nutritional contents in threes species of chili pepper namely *Capsicum frutescence L., Capsicum annuum var. annuum, and Capsicum annuum* L. var *cerasiforme* are evaluated by proximate analysis. The methanol extract of the berries were subjected to phytochemical screening, estimation of total phenolic, flavonoid and tannin contents were estimated by Folin-Ciocalteu, aluminum chloride colorimetric and Vanilline-HCl methods respectively. The antioxidant, antibacterial and antidiabetic activities were evaluated by the DPPH method, dis-diffusion and α-amylase inhibition assay methods respectively.

2. Materials and methods

2.1 Chemicals and equipment

Methanol, acetone, n-hexane, ethyl acetate of Fisher Scientific and dimethyl sulphoxide of Merk brand were used. Folin-Ciocalteu reagent (FCR), α-amylase enzyme, acarbose of Himedia were used. The chemicals like gallic acid, ascorbic acid, quercetin, (Himedia) and 2, 2- diphenyl-1- picrylhydrazyl (Tokyo chemical industries co. ltd), $NaNO_2$, $AlCl_3$, KOH, NaOH of Fisher

Scientific company were used. Reagents and solvents used during phytochemical screening analysis were prepared in the chemicals provided in the laboratory.

Different borosilicate glassware, electrical grinder, mortar and pestle, distillation flask, Soxhlet extractor, digital weighing balance, hot air oven, rotatory evaporator with a water bath, spectrophotometer (BioTEk Energy LX multimode reader), hot water bath, micropipettes (cleaver), tips incubator, and 96- well plate reader instrument.

2.2 Methods

2.2.1 Sample collection and preparation of methanol extract

The ripen berries of three capsicum were collect from Kathmandu and Ramechhap districts of Nepal. The plant species were identified at the National Agricultural Research Institute, Khumaltar, Lalitpur using the herbaria of the plants. The berries were rinsed with distilled water and go through proximate analysis. The berries were separately cut down into small pieces and air-dried for four weeks, and ground into a fine powder using a mechanical grinder. All the three samples were extracted using a Soxhlet apparatus with 80% methanol. The crude extracts were concentrated by using a rotary evaporator and stored in a refrigerator at 4ºC for further analysis.

2.2.2 Phytochemical analysis

Presence of different phytochemicals in the extracts of chili pepper as performed by using a standard protocol (Bora et al., 2019; Tiwari et al., 2020). The test was performed for the presence of flavonoids, polyphenols, alkaloids, reducing sugars, quinones and glucosides.

2.2.3 Proximate analysis

The proximate analysis was performed for the quantification of total moisture, total ash, crude fat, protein, crude fiber and total carbohydrates using standard methods (Ganogpichayagrai & Suksaard, 2020; Sharma & Giri, 2022).

2.2.3.1 Determination of moisture content

The moisture content in the chili samples was determined by hot air oven method. 5 g of the fresh dry samples were heated carefully in a crucible at 100 ±2ºC for about 6 hours and cooled in a desiccator for half of an hour. The loss of weight is taken as a measure of the moisture content of the different samples.

The moisture content was calculated by:

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

Where, $W_1 =$ weight of the sample was taken.

2.2.3.2 Determination of total ash content

Exactly 25 g of dried samples were taken in a tared crucible and heated at 100ºC to evaporate all the water. Few drops of olive oil were added and heated slowly in a muffle furnace at 525ºC to

get the white ash. The crucible was cooled and weights were taken. The process was performed in triplicate and the total ash content in the chili fruit was calculated by using the formula:

$$\text{Total ash (\%)} = \frac{W_1 - W}{W_1} \times 100$$

W_1 = weight of the sample taken

2.2.3.3 Determination of crude fat

Nearly 2 g of the samples were taken in thimble of Whatmann No. 1 filter paper and the open end was clogged with a cotton plug. The loaded thimble was put in a Soxhlet apparatus and extracted by using petroleum ether. The solvent was removed by evaporation and the crude fat content was calculated as:

$$\text{Crude fat (\%)} = \frac{W_2}{W_1} \times 100$$

Where W_2 = Weight of fat

W_1= Weight of the sample taken

2.2.3.4 Determination of protein

The amount of protein content in the three samples of chili pepper was estimated by the Kjeldahl's method. The protein content was calculated by using a nitrogen conversion factor of %NX6.25 as analyzed by the Kjeldahl's method.

2.2.3.5 Determination of crude fiber

Two grams of defatted samples were taken in Erlenmeyer flask with 200 ml of 0.2N H_2SO_4 and boiled for 30 minutes with a lid at the mouth of the flask. It was washed with water to remove residual acid and transferred into another flask and boiled again with 200 mL of 0.32N NaOH, filtered and washed. The residue was transferred into a gooch crucible and washed with 95% ethyl alcohol and dried in a hot air oven at 100 $\pm$ 1 ℃. Finally, the content was cooled and weighed and heated in a muffle furnace at 600 ± 20 ℃ for 3-4 hours for ashing.

The crude fiber was calculated by the formula,

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

Where,

W_1 = weight of the gooch crucible with content before ashing

W_2 = weight of the gooch crucible with ash

W = weight of the defatted sample taken

2.2.3.6 Estimation of carbohydrates

Total carbohydrates present in each sample of chili were calculated by subtracting the total protein, crude fat, moisture content, and ash content from 100 g samples.

Carbohydrate= 100-(%protein+%crude fat + % ash) + %moisture content)

2.2.4 Estimation of total phenolic and flavonoid content

The total phenolic contents (TPC) and total flavonoid contents (TFC) present in each of methanol extracts of three chili samples in three chili samples were determined by the Folin-Ciocalteu and aluminum chloride colorimetric method respectively (Phuyal et al., 2020; Singleton, Vernon et al., 1999). For TPC determination, 20 µL of standard gallic acid of 10, 20, 30, 40, 50, 60, 70, and 80 µg/mL concentrations were loaded in the wells of a 96-well plate in triplicate. Similarly 20 µL of plant extracts of 5 mg/mL were also loaded in triplicates. To each of the test samples, 100 µL of FCR (diluted to 1:10 with DW), and 80 µL of 1M Na_2CO_3 were added to each of the samples by using a multichannel pipette. The mixture was shaken slightly and incubated for 30 minutes in the dark and the optical density was recorded against a blank at 765 nm. The total phenolic content in each of the sample was calculated by using the standard gallic acid curve and expressed as mg GAE/g. For the determination of TFC, 130 µL of quercetin solutions of 10, 20, 30, 40 50, 60, 70, and 80 µg/mL quercetin was loaded into the wells of a 96-well plate in triplicate. Similarly, 20 µL of plant extract (5 mg/mL) and 110 µL of DW for each of the chili samples were loaded in triplicates. To each of the bores 60 µL of ethanol, 5 µL of $AlCl_3$ and 5 µL of CH_3COOK solutions were added by using a multichannel pipette and incubated for 35 minutes in the dark. Then, the optical density of the solutions were measured at 415 nm against blank by using a microplate reader. The TFC was calculated from the standard curve of quercetin and expressed as mg QE/g by regression analysis.

2.2.5 Determination of antioxidant capacity

The antioxidant capacity of methanol extracts of three chili samples was evaluated by DPPH free radical scavenging method taking quercetin as a positive and 50% DMSO as negative control (Khanal, 2023; Niroula et al., 2019; Shahidi & Ambigaipalan, 2015). Briefly, the plant samples as well as the positive control were diluted into 20, 10, 5, 2.5, and 1.25 µg/mL from the stock solutions by serial dilution. Aliquots of 100 µL of the extract, positive and negative controls were loaded into the wells of a 96-well plate in triplicates. To each of the test samples, 100 µL of 0.1 mM DPPH solution were added and incubated for 30 minutes in the dark. Then, the absorbance was taken at 517 nm using a microplate reader. The capability to scavenge the DPPH radical was calculated by

$$\text{DPPH scavenged (\%)} = \frac{A_{\text{Control}} - A_{Sample}}{A_{\text{Control}}} \times 100$$

Where,

A = absorbance of the control and sample.

2.2.6 α-Amylase inhibitory activity

The α-amylase inhibition activity was evaluated by the 2-chloro-4-nitrophenyl-α-D-maltotrioside ($CNPG_3$) method taking acarbose as a positive control (Timilsina et al., 2023). Aliquots of 20 µL of the test samples/acarbose of different concentrations were mixed with 80 µL of pancreatic porcine- α-amylase (PPA) of 1.5U/mL and incubated for 10 minutes in the dark at

37 °C. Then, 0.5 mM of $CNPG_3$ in phosphate buffer was added into each of the cells in the microplate and incubated for 15 minutes. After incubation, absorbance was measured at 405 nm using a microplate reader.

$$\% \, Inhibition = \frac{An - As}{An} X100$$

Where,

A_n is the optical density of the negative control with 30% DMSO and A_s is the optical density of the test/positive control.

2.2.7 Antibacterial activity

The antibacterial susceptibility of the methanol extracts of fruits of three chili pepper was evaluated against both gram negative and gram positive bacteria (**Table 1**) by the disc-diffusion method (Murray et al., 2007; Wasihun et al., 2023).The pre-incubated sterile Muller-Hinton petri plates were taken and wells of 6 mm diameter were punched using a cork borer. Aliquots of 20 µL of the test solutions, negative control (50% DMSO), and positive control (chloramphenicol) were loaded using a micropipette. The plates were covered with a lid and incubated at 37ºC for 24 hours. On the next day, the petri discs were taken out and the clear zone around the sample were measured recorded as zones of inhibition.

Table 1: List of bacteria tested

S.N.	Name of species	Reference No.	Strains
1	*Staphylococcus aureus*	ATCC-25293	Gram-positive
2	*Escherichia coli*	ATCC-25922	Gram-negative
3	*Klebsiella pneumoniae*	ATCC-13883	Gram-negative
4	*Salmonella typhi*	ATCC-14028	Gram-negative

3. Results and discussion

3.1 Phytochemical screening

The results of phytochemical screening of methanol extracts of three samples of chili fruits are shown in **Table 2.**

The results of phytochemical screening of extracts shows the presence of flavonoids, reducing sugar, glucosides, and quinones in all of the samples whereas alkaloids and saponins are not detected. Nowadays phytochemicals in foods are considered as the functional food constituents that have preventive attributes for many diseases. The health benefits of these secondary metabolites depend on their purity and structural stability (Kumar et al., 2023).

Table 2: Results of phytochemical screening

S.N.	Phytochemicals	*Capsicum frutescence* L.	*Capsicum annuum var. annuum*	*Capsicum annuum* L. var *cerasiforme*
1	Alkaloids	–	–	–
2	Flavonoids	+	+	+
3	Reducing sugar	+	+	+
4	Glucosides	+	+	+
5	Quinones	+	+	+
6	Saponins	–	–	–
7	Polyphenol	+	+	+

Note: (+) presence of phytochemicals and (–) absence of phytochemicals

3.2 Proximate analysis

The results of the proximate analysis of three species of chili of Nepalese origin are shown in **Table 3**. In this study highest moisture content was found in *Capsicum annuum* L. var *cerasiforme* (81.59%) followed by *Capsicum annuum* var. *annuum* (74.43%) and the lowest moisture was found in the extract of *Capsicum frutescence* L. (61.34%). Water is the most abundant component in all of the veggies and fruits which play vital role in regulating pH, Temperature, and integrity of the tissues and cells in human body. The content of crude fiber is in reverse order that ranged from 16.43% to 6.26%. In this study, the crude protein content varied from 2.06% in *C. annuum L. var cerasiforme* followed by 4.78% in *C. frutescence* L. and the highest of 5.49 % was found in *C. annuum var. annuum.* The crude fat content was found 1.47% of maximum in *C. frutescence* L. to minimum of 0.56% in *C. annuum* L. var *cerasiforme.* The total carbohydrate and total ash content were found in the same order. The maximum carbohydrate of 13.59% in *C. frutescence* L. followed by 6.09% in *C. annuum* var. *annuum* and lowest in *C. annuum* L. var *cerasiforme* (8.07%) was observed. Similarly, total ash also varied from 2.39% in C. *frutescence* L. to the minimum of 1.44% in *Capsicum annuum* L. var *cerasiforme.* Similar to our study, Kefale et al. (2023) reported lower moisture levels and higher amounts of protein, fiber, and fats, likely due to the analysis of fully dried red pepper samples. However, in our study, we analyzed wet samples. The migration of inorganic ions to fruits from different parts of from various parts of the plant during ripening results higher ash content. The higher ash content in our results are lower than observed by Khan et al. (2019).

Table 3: Results of proximate analysis of chili fruits

Test Parameters	*Capsicum frutescence* L.	*Capsicum annuum* var. *annuum*	*Capsicum annuum* L. var *cerasiforme*
Moisture	61.34	74.43	81.59

Crude fiber	16.43	11.71	6.26
Crude protein	4.78	5.49	2.06
Crude fat	1.47	0.62	0.56
Carbohydrate	13.59	6.09	8.07
Total ash	2.39	1.65	1.44

3.3 Total phenolic and total flavonoid content

Folin-Ciocalteu reagent, a mixture of tungstate and molybdates works on the mechanism of oxidation-reduction reaction where the reduction of the mixture heteropolyphosphotungstates-molybdates by the phenolic compounds occurs resulting in the formation of blue-colored chromogen. The phenolic compound reacts with Folin-Ciocalteu only under the basic conditions adjusted by sodium carbonate solutions. Total phenolic content (TPC) of methanol extracts of three different capsicum species were expressed in terms of gallic acid equivalent (mg GAE/g dw) with a calibration curve of gallic acid. The results in **Table 4** shows the extract of *Capsicum frutescens* L. showed the highest (71.80 ± 3.36 mg GAE/g dw) TPC value followed by *Capsicum annuum* var *annuum***.** (38.00 ± 3.11 mg GAE/g dw) and lowest TPC content was exhibited by *capsicum annuum* L.var *cerasiforme* (6.59 ± 0.50 mg GAE/g dw). Phenolic compounds have been known to possess high antioxidant properties due to their free radical scavenging properties. It has been reported that extract containing a large amount of polyphenol content possesses a greater antioxidant activity. Although the quantitative determination of phenolic compounds in plant extracts is hampered by their structural complexity, diversity, nature of analytical assay method, selection of standard, and presence of interfering substances (Rani et al., 2018).

On the total flavonoids content in three different capsicum species, extract of *Capsicum frutescence* L. showed the highest (22.38 ± 2.56 mg QE/g dw) TPC value followed by *Capsicum annuum* var. *annuum* (20.18 ± 1.20 mg QE/g dw) and lowest TPC content was exhibited by *Capsicum annuum* L. var *cerasiforme* (3.20 ± 0.43 mg QE/g dw) **Table 4**. The quantitative determination of flavonoid compounds in plant species are influenced by their structural complexity, diversity, nature of analytical assay method, selection of standard, and presence of interfering substances. Yet, obtained value is comparable to the previously reported values for total flavonoid content of ethanolic extract are 867.24 ± 53.87 mg QE/g dw for *Capsicum frutescence* L. varieties. Similarly, 1630.53 ± 86.96 mg QE/g dw for ethanolic extract of *Capsicum annuum* varieties (Olatunji & Afolayan, 2018).

3.4 Antioxidant capacity

The oxidant capacity of the methanol extracts of chili fruits were assessed by DPPH method which is easy and the results are shown in **Table** 4. Among the three samples tested, *C. frutescence* and *C. annuum* L.var *cerasiforme* were found to be more potent antioxidants than *Capsicum annuum* var. *annuum* had lower radical scavenging capacity. The half maximal concentration (IC_{50}) value ranged from 99.13 ± 3.55 for *C. frutescence*, followed by 100.75 ± 7.42 *Capsicum*

annuum L.var *cerasiforme* and the highest value was shown by *Capsicum annuum* var. *annuum* with the highest IC_{50} value of 186.73 ± 5.78μg/mL. The plant with greater flavonoid and phenolic content found to have higher antioxidant activities too. The obtained value are comparable to the previously reported IC_{50} value for the two plant species are 150.40 ± 8.07 μg/mL for *Capsicum annuum* var. *annuum* and 40 μg/mL for *Capsicum annuum* L. var *cerasiform.* Similar to our study, Red paprika ethanol extract was reported to exhibit a moderate antioxidant capacity on DPPH method with an IC_{50} value of 150.40 ± 8.07 μg/mL (J. S. Kim et al., 2011). The fresh chili extracts showed stronger antioxidant activity compared to the dried chili extracts obtained from a vegetable market in Kathmandu. The IC_{50} values for the fresh chilies ranged from 18.02 to 94.47 μg/mL, while the dried chilies had IC_{50} values ranging from 20.86 to 121.40 μg/mL (ThapaMagar & Shrestha, 2023).

Table 4: Results of TPC, TFC and IC_{50} values of methanol extracts of chili pepper

Name of plants	TPC (mgGAE/g)	TFC (mgQE/g)	IC_{50} (μg/mL) (Antioxidant)
Capsicum frutescence L.	71.80 ± 3.36	22.38 ± 2.56	99.13 ± 3.55
Capsicum annuum var. *annuum*	38.00 ± 3.11	20.18 ± 1.20	186.73 ± 5.78
Capsicum annuum L. var *cerasiforme*	6.59 ± 0.50	3.20 ± 0.43	100.75 ± 7.42

3.5 Antidiabetic activity

The alpha amylase inhibition capacity of chili extracts were measured by CNPG3 method and the results are shown in **Table 5**. The percentage inhibition of the enzyme was compared to that of acarbose. At the concentration of 5 mg/mL, three extracts showed the inhibitory capacity in the order as: *Capsicum annuum* var. *annuum (*11.51 ± 0.91%) followed by *Capsicum frutescence* L. (8.76 ± 1.20%), and the lowest inhibition was exhibited by *Capsicum annuum* L.var *cerasiforme (*4.06 ± 1.35%). Other studies also reported the good antidiabetic activities of chili (H. K. Kim et al., 2020; Watcharachaisoponsiri et al., 2016).

Table 5: α-Amylase inhibitory capacity (%) of different chili extracts

Name of plants	**Inhibition percentage**
Capsicum frutescence L.	8.76 ± 1.20
Capsicum annuum var. *annuum*	11.51 ± 0.91
Capsicum annuum L. var *cerasiforme*	4.06 ± 1.35
Acarbose	99.98 ± 1.12

3.6 Antibacterial activity

The antibacterial activities of three different extracts of genus *Capsicum* were performed towards two- Gram-positive and two-Gram negative bacteria. It has been observed that all of them have no inhibition towards *Klebsiella pneumonia, Salmonella typhi, Escherichia coli,* and *Staphylococcus aureus.*

4. Conclusions

This study reveals the presence of significant phytochemicals in the pepper plant, which contribute to its key biological and therapeutic properties. Proximate analysis identified carbohydrates, proteins, fats, and fiber in the chili fruits commonly consumed in Nepal. The methanol extract of red pepper was found to contain a significant quantity of phenolic and flavonoid compounds and revealed strong DPPH free radical scavenging activity as well as notable α-amylase inhibition. The findings of this study support the use of red chili fruits as nutraceuticals, dietary supplements, and source of vital minerals with potential to reduce the risk of diseases linked to free radicals.

Conflicts of interest: Authors declare no conflicting interests.

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