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Original Article

Assessment of Phytochemicals, Antioxidant and Free Radical Scavenging Potential of Aqueous Methanol Extract of *Capsicum assamicum* Jubilee Purkayastha & L. Singh (Bhut Jolokia) fruits

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ABSTRACT

Objective: The aim of this study was to assess the phytochemicals, antioxidant and free radical scavenging potential of aqueous methanol extract of *Capsicum assamicum* Jubilee Purkayastha & L. Singh (Bhut Jolokia) fruits so as to establish the possible source of natural antioxidant for use in nutraceutical, pharmaceutical, cosmetic and food industries. **Methodology**: Phytochemicals such as total phenolics and flavonoids were assessed by Folin-Ciocalteu and aluminum chloride methods and results are expressed as µg gallic acid and quercetin equivalents g^{-1}, respectively. Capsaicin content was determined using spectrophotometer and expressed in percentage of chili powder. Antioxidant activity was estimated by the method of Prieto *et al* and expressed as ascorbic acid equivalent g^{-1} extract. Free radical scavenging capacity was determined by 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-Azino-bis (3- ethylbenzothiazoline 6-sulfonic acid (ABTS) and hydroxyl (OH) radical scavenging assays and expressed in term of IC_{50}. Ascorbic acid was used as standard. **Results**: Total phenolic content of extract as assessed by Folin-Ciocalteu reagent was estimated to be as high as 1842.01±6.98 µg gallic acid equivalents g^{-1} extract. Flavonoids content was determined to be 206.73±3.05 µg quercetin equivalents g^{-1} extract. Capsaicin content was as high as 6.07%. Total antioxidant capacity was determined to be 108.29 ±0.87 µg ascorbic acid equivalents g^{-1} extract. IC_{50} values of extract and ascorbic acid for DPPH radical scavenging potential were 74.42±2.31 and 5.45±0.45 µg/ml, respectively. IC_{50} values of extract and ascorbic acid for ABTS radical scavenging potential were found to be 25.00±0.32 and 9.15±0.15 µg /ml, respectively. Similarly, hydroxyl radical scavenged by 50% at concentrations of 2.15±0.04 µg/ml and 1.95±0.89 µg/ml of extract and ascorbic acid, respectively. **Conclusion**: Our results indicated very high amount of phenolic compounds, flavonoids and capsaicin in the extract. These phytochemicals are well known for their antioxidant activities. Moreover, good antioxidant capacity and free radical scavenging potential were reflected in the current investigation. Therefore, *Capsicum assamicum* Jubilee Purkayastha & L. Singh is a potential candidate for exploitation as a source of natural antioxidant for dietary supplements and / or for pharmaceutical /cosmetic uses. *Capsicum assamicum* could also be cultivated on a large scale as it is endemic to the region which further substantiate the feasibility and potentiality of the same

Keywords: Hottest chili; *Capsicum assamicum*; phytochemicals; antioxidant; free radical. scavenging potential.

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1. INTRODUCTION

The Chili or Capsicum is believed to be the oldest domesticated plant genera dated back to 7000 years based on archaeological data[1]. Capsicum species are cultivated as vegetable and condiment crops worldwide. Chili fruits are rich sources of secondary metabolites such as carotinoids, vitamins, phenolics and capsaninoids that are beneficial to human health.[2,3]

Capsicum assamicum Jubilee Purkayastha & L. Singh (Bhut Jolokia) is endemic to north eastern region of India. It is extensively cultivated in the region, predominantly in the states of Assam, Nagaland and Manipur and to some extend in Arunachal Pradesh, Meghalaya and Mizoram. It is also cultivated in the north eastern region of Bangladesh.[4] It has been associating with the ethno-agricultural activities of people in the region. It is known by various local names such as : *Bhut Jolokia*, in Assamese, U- morok in Manipuri, Naga King Chili in Nagaland. Nagaland state Government obtained the Geographical Indication of Goods tag (Registration & protection) for Naga King Chili from Government of India in 1999. Above names are derived from the characteristics of its fruits such as pungency, pod size and the appearance of the plant. It is cultivated and consumed fondly in the region as spice and / or eaten raw as appetizer along with staple food/rice.

Bhut Jolokia was first reported as the hottest chili from Defence Research Laboratory Tezpur with 855,000 Scoville Heat Units (SHUs). The hottest chile pepper on record then was the *C.chinense* Jacq. cultivar Red Savina with heat level of 577,000 SHUs. This finding pushed back the then hottest chili of Maxico. Initially, this fact was questioned in the scientific forum. New Mexico State University reported heat level of 1,001,304 Scoville Heat Units (SHUs) [6] in Bhut Jolokia, almost double the SHUs of Red Savina. *Bhut Jolokia* was recognized as world's hottest chili by Guinness World Record.[7] Ever since this chili has been gaining importance among the scientific community.

Capsicum species are incorporated into medicinal preparations in the ancient literature around the World.[8] In Indian systems of medicine including Ayurveda, Siddha and Unani[9,10,11] the dietary spices form important ingredients for treating chronic as well as acute diseases. *Bhut Jolokia* is one such spice which is used by ethnic groups in north eastern India as remedy against ailments such as asthma, gastritis, chronic indigestion problems, etc. Hot infusions of mature fruits for relieving toothache and muscle pain, paste of tender leaves is applied as thin covering over boils for easy removal of pus.[12] Fresh fruit has unique aroma which is very refreshing and palatable and preferred as appetizer in small quantity along with the meal. It is also preferred over other spices for added taste and flavor to many local dish of the region: such as preparing soup, pickle, spice, soft drink, candies, etc. Oleoresin extracted from fruits is in high demand as it is the most heavily and frequently consumed spices throughout the world. Oleoresin has its application as non lethal weapons for self defence and containing low intensity conflicts as well.

Bhut Jolokia has become a plant of scientific attention in recent time due to the very high capsaicin content and of its multi-dimentional uses. Secondly, capsaicin is a potential molecule for the development of new generation of analgesic/anti-inflammatory medicines.[13] Thirdly, it has application in food industry as natural food additives, preservative and colouring agent. The present study was designed to assess its phytochemicals, antioxidant and free radical scavenging capacity with the intend to explore as natural and sustainable based free radical scavengers or antioxidant source.

2 MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent**,** 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-Azino-bis (3- ethylbenzothiazoline 6-sulfonic acid (ABTS), Ascorbic acid, Gallic acid, capsaicin were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals or reagents were of analytical grade from Himedia, Merck – Mumbai, India.

Plant material

Bhut Jolokia fruits were collected from local market at Senapati, Manipur, India. Seedlings were then raised in a nursery bed, transplanted onto experimental plots. Thus Chili plants were cultivated in our experimental plot at Tezpur (longitude 91° 48' E and latitude of 26° 38' N). For our investigation, mature fruits collected were the first batch of the first year fruiting in 2016. Fruits are sub-conical to conical in shape, orange to red in colour, dented surface with rough/spiky texture, extraordinary hotness/pungency and uniquely refreshing and palatable taste/ flavor.

Preparation of extract

Bhut Jolokia fruits were dried in an Oven (NSW-143, India) at 40° C for 72 h and ground using a mechanical grinder. 100 g of ground sample was extracted in 1000 ml of aqueous methanol (1:4) by cold maceration for 48 h with intermittent shaking. Extraction was repeated and the resultant extracts were pooled and filtered using Whatman No.1 filter paper. Filtrate was then concentrated under reduced pressure in a rotary vacuum evaporator (RV10 Control, IKA, Germany), which was then air-dried to a constant weight at room temperature (referred as extract). The brownish extract yield (27.97%) stored at -20°C till analyses.

Phytochemical analyses

Test extract was dissolved in methanol to make a concentration of 2 mg/ml and then diluted to series of concentrations for phytochemical analyses, antioxidant and free radical scavenging assays. Standard compounds were used for comparison in all the assays.

Total phenolic content

Total phenolic content (TPC) was determined by the method of Singleton & Rossi[14] using Spectrophotometer (UV-1,

Thermo, USA) using gallic acid as reference. The TPC was calculated from gallic acid standard curve. The TPC was expressed as µg of gallic acid equivalents (GAE) per g of extract. The test was performed in triplicates.

Total flavonoids content

Total flavonoid content (TFC) was estimated using aluminum chloride colorimetric assay[15,16] with quercetin as standard. TFC was determined at 510 nm in spectrophotometer and expressed as µg quercetin equivalent per g extract. Experiments performed in triplicates.

Capsaicin content

Capsaicin content was estimated spectrophotometrically at 280 nm and the concentration of which was determined from standard curve of capsaicin. Capsaicin content is expressed in percentage of dry chili powder.

Antioxidant Assays

Total antioxidant capacity

Total antioxidant capacity was estimated by the method described by Prieto *et al.*[17]. Total antioxidant capacity was determined from ascorbic acid standard curve and expressed as µg ascorbic acid equivalent per gram extract. The test was performed in triplicates.

DPPH radical scavenging assay

DPPH radical is measured by a UV spectrophotometer at 517 nm using the method described by Brand-Williams *et al.*[18]. Ascorbic acid was used as reference standard. Lower the absorbance higher the radical scavenging capacity and expressed as IC_{50}. IC_{50} values were the effective concentrations at which DPPH radicals were scavenged by 50%. DPPH radical scavenging activity was calculated as follows:

DPPH radical scavenged (%) = [(Abs control - Abs sample) /Abs control] × 100

The test was carried out in triplicates.

ABTS radical scavenging assay

ABTS (2 mM) and potassium persulphate (970 mM) were prepared in distilled water. 200 µl of potassium persulfate and 50 µl of ABTS were mixed and used after 2 h. This preformed ABTS radical cation in the solution was assayed according to Shirwaikar et al.[19] Ascorbic acid was used as reference. Radical scavenging activity was calculated as follows:

ABTS radical scavenged (%) = [(Abs control - Abs sample) / Abs control] × 100

The experiment was performed in triplicates.

Hydroxy radical scavenging assay

Hydroxyl radical scavenging capacity was measured according to the modified method of Halliwel *et al.*.[20] Ascorbic acid was used as reference. Hydroxyl radical scavenging capacity was calculated as follows:

Hydroxyl scavenged (%) = [(Abs control - Abs sample) / Abs control] × 100

The experiment was performed in triplicates.

3. RESULTS

Total phenolic content (TPC) in the extract was assessed by Folin Ciocalteu reagent and result is expressed as µg gallic acid equivalents per gram extract with reference to gallic acid. The result is shown in Table 1. Flavonoid content (FC) was determined by aluminum chloride method and expressed as quecetin equivalents in mg g^{-1} extract as shown in Table 1. Total antioxidant capacity (TAC) was determined by phosphomolybdate method and expressed as µg ascorbic acid equivalents g^{-1} extract, while extraction yield is given in percentage of dried chili powder in Table 1.

Extract and ascorbic acid scavenged DPPH radical in a concentration –dependent manner (Figure 2 A & B) with IC_{50} values of 74.42±2.31 and 5.45±0.45 µg/ml, respectively (Table 2). Extract and ascorbic acid scavenged ABTS radical in a concentration-dependent manner (Figure 2 C& D) with IC_{50} values of 25.00±0.15 and 9.15±0.15µg/ml (Table 2). Similarly, hydroxyl (OH) scavenged was also concentration dependent (Figure 2 E&F) and by 50 % at a concentration of 2.5 µg/ml and 1.85 µg/ml of extract and ascorbic acid, respectively (Table 2).

Table 1: Total phenolics, flavonoids, capsaicin, antioxidant capacity and yield of aqueous methanolic extract of *C. assamicum* Jubilee Purkayastha & L. Singh fruits.

Extract	Yield (%)	Capsaicin (%)	Total phenolics (µg GAE /g extract)	Total flavonoids (µg QE/g extract)	Total antioxidant capacity (µg AAE/g extract)
Aqueous methanol	27.97±0.08	6.07±0.05	1842.01 ± 6.98	206.73 ± 3.05	108.29 ± 0.87

Each value represented as mean ± SD (n = 3).
GAE; Gallic Acid Equivalent, QE; Quercetin Equivalent,
AA; Ascorbic Acid Equivalent

Table 2: IC_{50} values of *C. assamicum* Jubilee Purkayastha & L. Singh fruit aqueous methanol extract, ascorbic acid and gallic acid against free radicals.

	IC_{50} value (µg/ml)		
Extract/reference	DPPH radical	ABTS radical	Hydroxyl radical
Aqueous methanol	74.42 ±2.31	25.00 ±0.31	2.15 ±0.04
Ascorbic acid	05.45 ±0.45	09.15 ±0.15	1.95 ±0.89

Each value in the table is represented as mean ± SD (n = 3).
$IC_{50,}$ inhibition concentration

4. DISCUSSION

Numerous disorders or diseases are related with oxidants and free radicals. Therefore, agents that counter their effects definitely are beneficial either in health or disease. Antioxidants are ascribed with such properties. Exogenous intake of natural antioxidants either in the form of extracts or their chemical constituents are very effective to prevent the damage caused by oxidative stress.[21] They exert their actions either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms.[22] There has been a renewed interest in recent years on antioxidant studies of various crops, vegetables or plant base products.

Bhut Jolokia is an important agricultural crop, not only because of its economic importance, more importantly for its nutritional values and its constituents such as capsaicinoids

(the pungent principle) vitamins, carotinoids, etc. Recently, on many platforms, traditional and indigenous food resources are seen as viable options to meet nutritional security of the rural and create demand among urban populations. In many cases underutilized food resources have higher nutrients content than globally known species or varieties commonly produced and consumed. Present study was designed to evaluate the antioxidant capacity and the content of total phenolic, total flavonoids and capsaicin of its fruits. Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of substances as substances with low antioxidant activity *in vitro* will probably show little activity *in vivo*.[23] Moreover, *in vitro* antioxidant potential screening is useful and substantiated as well as *in vivo* studies are not always recommended due to several reasons such as ethical issues, cost effective issues, etc.

Plant materials rich in phenolics are increasingly being used in the food industry because of its ability to retard oxidative degradation of lipids thereby retaining the quality and nutritional value of food.[24] Due to their hydroxyl groups; phenolic compounds confer antioxidant or free radical scavenging capacity. Phenolic compounds derived from phenylalanine and tyrosines occur ubiquitously in plants and are diversified.[25] The aqueous methanolic extract exhibited the highest total phenolics content (data other than aqueous methanol extract not shown). Total phenolics content in the extract was found to be high as determined from gallic acid standard curve. Phenolic content was determined to know the nature of the constituents in the extract. Total phenolic content of the extract was estimated to be very high and thus very high or potent phenolics might have been extracted into aqueous methanol extract.

Phenolic compounds of plants fall into several categories and flavonoids, among others, have potent antioxidant activities[23] and therefore are thought to have positive effects on human health. Studies on flavonoidic derivatives have shown a wide range of activities such as antibacterial, antiviral, antiinflammatory, anticancer, and anti-allergic.[26, 27] Flavonoids have been shown to be highly effective scavengers for most oxidizing molecules, including singlet oxygen, and various free radicals[28] implicated in several diseases. Flavonoids content was determined so as to have an idea of its content in the extract and is expressed as quercetin equivalent g^{-1} extract from quercetin standard curve. Capsaicin, forms part of capsaicinoids, is known for its antioxidant potentency and pharmacological activities such as pain relieving, antiplatelet effect, antidiabetic effect, gastrointestinal benefits, hepatoprotective, etc. Capsaicin content was also estimated to be as high as 6% of chili powder.

Total antioxidant activity was carried out so as to understand the antioxidant capacity of the extract in the study. Phosphomolybdenum method gives reliable results with variety of samples such as plant lipid-soluble extracts, vegetable oils, butter, pharmaceutical and cosmetic preparations, human serum, etc. However, it gives the total antioxidant capacity of a sample irrespective of nature and mechanism of actions of drugs[17] In the present study, aqueous methanolic extract was found to exert strong capacity for reduction of phosphomolybdate from Mo (VI) to Mo (V) and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 765 nm. The total antioxidant capacity in quantitative term is expressed as ascorbic acid equivalent g^{-1} extract. Many phenolics, such as flavonoids and tannins are reported with strong antioxidant capacity coupled with the ability to donate hydrogen atom to radicals rapidly.[29, 30] Therefore, results of our study suggested that phenolic acids, flavonoids and capsaicin may be the major contributors for the strong antioxidant activity that was detected in the study.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical, pink in color which turns yellow when scavenged. Decrease in absorbance at 517nm indicated that free radical is scavenged. DPPH radical scavenging activity of both extract and ascorbic acid compound was found to be in similar trend in concentration dependent manner (Figure 2 A&B) with IC_{50} values of 74.42±2.31 µg/ml and 5.45±0.45 µg/ml (Table 2), respectively. This activity is strong if it is to be compared with other plant extracts as per literature reports. DPPH radical scavenging activity of extract could be due to the presence of polyphenols, flavonoids and capsaicin. Ascorbic acid is well known for its free radical scavenging potential. ABTS radical scavenging assay involves a method that generates a blue/green ABTS+ chromophore via the reaction of ABTS and potassium persulfate. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured by spectrophotometer. ABTS scavenging activity of both extract and ascorbic acid was found to be in similar trend which was concentration dependent (Figure 2 B&C), with IC_{50} values of 25.00±0.31 and 9.15±0.15 µg/ml (Table 2), respectively. Hydroxyl radical is the most reactive of ROS (e.g., hydroxyl radical, hydrogen peroxide and singlet oxygen), which induces severe damage in the adjacent biomolecules[31], which can cause oxidative stress to DNA, lipid and proteins[32]. Hydroxyl radicals, constantly generated in human body, may play an important role in tissue injury at the sites of inflammation in oxidative stress related diseases. In the present study, hydroxyl radicals, generated by fenton's reaction, attack deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA), upon reacting with thiobarbituric acid yield pink chromogen that is measured at 532 nm. Added extract to the reaction mixture found to reduce the absorbance indicating the removal of hydroxyl radical. The hydroxyl radical scavenging capacity of the extract and ascorbic acid were in concentration dependent (Figure 2 E&F) and the IC_{50} values of which were found to be 2.15±0.04 and 1.95±0.89 µg/ml

(Table 2), respectively. The exhibited scavenging potential was high. The observed activities could be due to the presence of above detected phytoconstituents in the extract.

Fig 1: *C. assamicum* Jubilee Purkayastha & L. Singh plant with fruiting and maturation (A), ripening (B), batch of mature & ripened fruits(C).

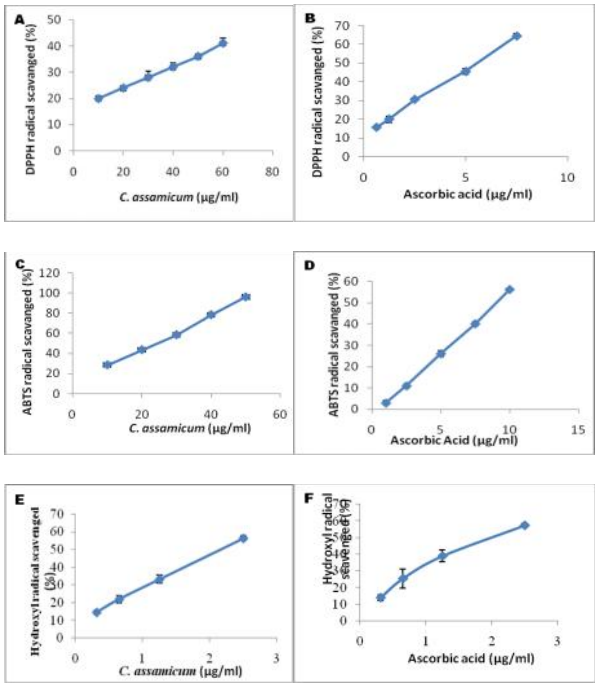

Fig 2: Radical scavenging activities of *C. assamicum* Jubilee Purkayastha & L. Singh fruit aqueous methanol extract and ascorbic acid at different concentrations. Each value represents an average of triplicates.

(A) Extract on DPPH, (B) Ascorbic acid on DPPH, (C) Extract on ABTS, (D) Ascorbic acid on ABTS, (E) Extract on hydroxyl, and (F) Ascorbic acid on hydroxyl.

5. CONCLUSION

Stress has become part of our everyday life today. If not manage in time, chronic stress can lead to a variety of stress-related disorders or ailments. Therefore, there is an ever increasing demand for natural antioxidants either to prevent or manage stress related disorders. Increasing demands have provoked great efforts for finding newer and potential free radical scavengers from plants. The present work was undertaken to assess the phytochemicals, antioxidant potency and free radical scavenging capacity of extract of *C. assamicum* Jubilee Purkayastha & L. Singh (*Bhut Jolokia*) fruits. *Bhut Jolokia* could be a good candidate for exploration as natural antioxidant source as our data implicated very high amount of beneficial phytochemicals such as phenolics, flavonoids and capsaicin, which are well known for their antioxidant activities and other biological activities. Moreover, potent antioxidant and free radical scavenging activity detected by various methods further substantiated the possible health benefit of *Bhut Jolokia* fruits. Therefore, consumption of *Bhut Jolokia* fruits could supply a substantial amount of the required antioxidants to promote health, prevent diseases and treatment of ailments thereby providing health security. Therefore, there is prospect of cultivating *Bhut Jolokia* on a large scale as it is endemic to the region making it more potential candidate for exploitation as a source of natural antioxidant for dietary supplements and / or for pharmaceutical /cosmetic uses. Isolation and characterization of bioactive components which are responsible for antioxidant and antiradical activities could also be undertaken.

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