Journal of Scientific and Innovative Research 2024; 13(4): 49-54
Available online at: www.jsirjournal.com

Research Article

ISSN 2320-4818
JSIR 2024; 13(4): 49-54

Received: 10-07-2024
Accepted: 29-12-2024
DOI: 10.31254/jsir.2024.13401

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Phytochemical screening and *in vitro* evaluation of antioxidant & antimicrobial activity of methanolic crude extract of fifteen varieties of pulses developed in Bangladesh

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Abstract

From a long time, certain plants have been utilized as a source of biologically active substances as well as a means of treating a variety of newly emerging or fatal diseases like cancer. In this study, the selected different varieties of pulses recently developed by the Pulse Research Center, Bangladesh such as BARI Masur-3 & 8, BARI Mung-6 & 8, BARI Chola 5 & 10, BARI Khesari-2 & 3, BARI Mash-3 & 4, BARI Arhar, BARI Faba beans, BARI Felon-1 and BARI Motor-2 & 3 were subjected to phytochemical screening and in vitro assay to explore their medicinal values. In the case of an antioxidant assay, after running with different solvent systems, almost all the extracts on the TLC plate, followed by treatment with DPPH, exhibited fluorescence under a UV detector, indicating the presence of antioxidant compounds. Further antioxidant capacity was investigated using the DPPH free radical scavenging assay. The proximity of the SC50 values of the crude extracts (BARI Mash-3, 74.65µg/mL; BARI Chola-10, 76.48µg/mL; and BARI Faba bean, 95.50µg/mL) to those of standard ascorbic acid (16.61 µg/mL) indicated strong free radical scavenging activity. Moreover, crude extracts of pulses demonstrated antimicrobial activity against two pathogenic bacteria, Staphylococcus aureus and Escherichia coli. Furthermore, a high concentration of phenols, flavonoids, and tannins was found in pulse extract, which supports their strong antioxidant and antibacterial activity. It can be concluded that this study revealed the traditional medicinal values of these pulses against infectious diseases, cancer, etc. Further investigation is needed to explore the other medicinal values and the isolation of bioactive compounds for their pharmacological applications.

Keywords: Pulse, Antimicrobial, Antioxidant, Phytochemicals, Medicinal values.

INTRODUCTION

For thousands of years, a wide range of physiologically active phytochemicals have been extracted from medicinal plants. Traditional practitioners use the plant's derived crude extracts or its purified phytochemicals extensively to cure a variety of infectious diseases [1,2]. Therefore, these are the fields of interest for additional investigation to extract and isolate diverse biologically active phytochemicals using the solvent extraction method [3]. Recently, natural products have provided numerous prospects for the development of distinct medicinal compound in order to the remarkable abundance of chemical variety. In our country, many natural plants remain understudied. Hence, scientific studies are required for the investigation of their micro and macro molecules, which possess a crucial function in the management of diverse ailments [4]. Over 6,500 plants are considered to be indigenous to Bangladesh, in excess of 500 plants having medicinal significance, from where 250 plants are commonly utilized in the preparation of medications for medical purposes [5]. Medicinal products from plats in Bangladesh are both affordable and easily accessible. Research on medicinal plants and ethnomedicine have been conducted in various regions of Bangladesh during the last 20 years, attracting remarkable attention from the government and pharmaceutical industry [6]. For at least 10,000 years, people have been consuming pulses as a nutritional food in the world [7]. More than 80% of the people in our country consume pulse as a food source, but many of them do not know the nutritional benefits and the health benefits. Many researchers have studied pulses and reported their nutritional value and health benefits.

According to the FAO dry edible seeds comprising legumes with minimal fat content is known as pulse [8]. It has been reported that Pulses possess fewer calories and lipids and more in macronutrients including proteins (21–26%) and carbs [9]. Previous researchers reported that the amino acids in pulses were responsible for their higher nutritive values [10]. It is actually feasible to make use of pulse proteins in foods without gluten like muffins and edible bio-disposable films [11]. A number of bioactive substances inside the pulses are not considered nutrients but exerts a significant metabolic effect on the human body upon consumption [12]. Phytochemicals (mainly polyphenols, flavonoids, tannins, resins, and phytosterols), dietary fibres and resistant starches stimulate the application of extensively in dietary products [13]. There is an abundant amount of potassium and a good quantity of calcium, magnesium, and phosphorus phytoconstituents present in pulses as well as minor quantity of heavy metals (selenium, copper, zinc, iron and manganese) and vitamins A and C [14]. Researchers reveled that pulses contain the strong free radical scavenging compounds called antioxidant compounds [15]. As oxidative stress continuously damages cells in the body leading to diverse group of diseases like aging, cancer, myocardial infarction, plants possessing phytochemicals (Polyphenols, tannins, resins, alkaloids, flavonoids, and terpenoids) are the emerging bioactive substances capable to prevent oxidation inside the human body [16]. Several varieties of pulses developed by Bangladesh Agriculture Research Institution (BARI) were selected to addressing the medicinal values (Table 1). BARI is the autonomous institution of the Ministry of Bangladesh in which Pulse Research Centre (PRC) is a sector of BARI which has developed a new breeding lines and varieties [17]. We selected the BARI released varieties of pulses like BARI Mash 3 & 4 (*Vigna mungo*), BARI Chola-5 & 10 (*Cicer arietinum*), BARI Mung-6 & 8 (*Vigna radiata*), BARI Masur-3 & 8 (*Lens culinaris*), BARI Khesari-2 & 3 (*Lathyrus sativus*), BARI Arhar (*Cajanus cajan*), BARI Felon-1 (*Vigna unguiculata*), BARI Motor-2 & 3 (*Pisum sativum*), and BARI Faba bean (*Vicia faba*) because of their higher production, pest resistance capacity, short lifespan, and because they are popularly consumed by the vast majority of the people. In Bangladesh, a lot of people consume pulses daily without knowing its nutritional and medicinal values. There are several varieties of pulses released by BARI that have not yet been analyzed for their medicinal values as well as their biological activities. If it is possible to find out the whole biological activity of pulses that are available in Bangladesh, it will increase consumption of pulses as medicinal food. Therefore, the research objective was to investigate the medicinal value of pulses. To determine the antioxidant values, the DPPH free radical scavenging method and the antimicrobial activity test were performed using disc diffusion technique. This study is also a leading point to encourage future researchers to investigate and isolate the lead compounds for their effective therapeutic applications. The incisive information from this study will be helpful for further investigation of other medicinal values and will increase consumption also.

Table 1: BARI developed varieties of pulse consumed in Bangladesh

Common Name	BARI developed Name	Scientific Name
Lentil	BARI Masur	*Lens culinaris*
Mung bean	BARI Mung	*Vigna radiata*
Chickpea	BARI Chola	*Cicer arietinum*
Grass pea	BARI Khesari	*Lathyrus sativus*
Black Gram	BARI Mash	*Vingan mungo*
Arhar	BARI Arhar	*Cajanus cajan*
Faba Bean	BARI Faba Bean	*Vicia faba*
Cowpea	BARI Felon	*Vigna unguiculata*
Field Pea	BARI motor	*Pisum sativum*

Figure 1: BARI developed variety of pulses. (a) Mash-3, (b) Mash-4, (c) Chickpea-5, (d) Chickpea-10, (e) Mung Bean-6, (f) Mung Bean-8, (g) Mashur-3, (h) Mashur-8, (i) Khesari-2, (j) Khesari-3, (k) Motor-2, (l) Motor-3, (m) Felon-1, (n) Arhar and (o) Faba Bean.

MATERIALS AND METHODS

Chemicals and Reagents

All reagents used to perform the experiments were analytical grade. The reagents such as methanol (Sigma-Aldrich, 99.99%), ethanol (Sigma-Aldrich, 99.5%), gallic acid (Sigma, USA), quercetin (Sigma, USA), ascorbic acid (Merck, Germany), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma, USA), $AlCl_3$ (Loba, India), NaOH (Loba, India), Na_2CO_3 (Loba, India), Folin-Ciocalteu (FC) reagent (Sigma Chemical Co. Ltd) were used.

Collection and Processing of sample

Fifteen varieties of pulse samples were collected from Pulses Research Centre of Bangladesh Agricultural Research Institute (BARI), Ishwardi, Pabna, Bangladesh in October, 2022. After collection, every sample was washed to get rid of dust and dirt from them. Then, all the samples were sun dried before grinding with the help of a grinder machine. To avoid cross- contamination, the grinder machine was cleaned properly before performing grinding procedure for separate samples.

Preparation of extract

At first, each 250 g powdered fifteen pulse samples was soaked in methanol (99.99%) for 15 days. Then, the mixture was shaken gently at a regular interval to mix the samples properly with the solvent. A clear filtrate was collected through filtration of the extract with cotton which subsequently filtered by filter paper (Whatman no 1) to eliminate any powdered particles. For the complete evaporation of solvent, a rotary solvent evaporator was used. Finally, the dried extract was preserved in a dried condition for subsequent analysis. The weight of different dried crude extracts of BARI Masur-3, BARI Masur-8, BARI Mung-6, BARI Mung-8, BARI Chola 5, BARI Chola 10, BARI Khesari-2, BARI Khesari-3, BARI Mash-3, BARI Mash-4, BARI Arhar, BARI Faba beans, BARI Felon- , BARI Motor-2, BARI Motor-3 were 5.34gm, 4.02gm, 5.76gm, 4.89gm, 6gm, 14gm, 5.103gm, 5.079gm, 4.84gm, 4.16gm, 4gm, 4.3gm, 5.1gm, and 4.8gm respectively.

Investigation of Phytochemicals

Quantification of Total Phenolic Content (TPC)

TPC of sample extracts was quantified according to Folin-Ciocalteu method with respect to standard gallic acid [18]. 5mL of 10% (v/v) aqueous Folin-Ciocalteu reagent was mixed separately with sample solution (0.5mL) and standard solution having range of concentration from 0.02 to 0.15 (mg/mL) in extraction solvent (methanol). After that, an equivalent of 4mL of 7.5% (w/v) Na_2CO_3 solution poured to each mixture. After homogenizing the mixtures in the individual test tubes for 15 seconds with a vortex, incubated at 40°C for 30 minutes. Simultaneously, a blank solution was made using all of the reagents without including sample extract or standard (gallic acid). By using UV spectrophotometer (set wavelength λ =765nm), absorbance of reaction mixture was recorded against a blank. TPC was demonstrated as (mg of GAE/gm of dry extracts) by using calibration curve of gallic acid.

Quantification of Total Flavonoid Content (TFC)

TFC of sample extracts was quantified by following the aluminium chloride colorimetric method where quercetin was used as a standard [18]. At first, 1mL of ethanolic sample solution, 4mL of distilled water and 0.3mL of 5% (w/v) $NaNO_3$ solution were mixed thoroughly. After 5 minutes, 0.3 mL of 10% (w/v) $AlCl_3$ was poured to the previous mixture and left for 1 minute. Volume of the final solution was adjusted up to 10mL by the addition of 2mL NaOH solution (1M) and distilled water. Subsequently, homogenization of the mixture conducted for 15 seconds before being left to react for another 30 minutes. By using UV spectrophotometer (set wavelength λ =510nm), absorbance of reaction mixture was recorded against a blank. The standard calibration curve was created using different doses of quercetin (0-1.0 mg/mL) to determine TFC that was demonstrated as (mg of QE/gm of dry extracts) by using the standard quercetin calibration curve.

Quantification of Total Tannin Content (TTC)

TTC of sample extracts was quantified by using Folin-Ciocalteu method with respect to standard gallic acid [18]. In brief, 0.1mL of ethanolic gallic acid solution (0.50, 0.40, 0.30, 0.20, 0.10 mg/mL) and sample extract solution (0.1mL) were poured individually in separate test tubes and 7.5mL distilled water was added to dilute the mixture. Thereafter, 1mL 35% Na_2CO_3 and 0.5mL FC reagent (10%) were poured to each test tube and volume of the final mixture was adjusted up to 10mL by the addition of distilled water. Simultaneously, a blank solution was prepared by using all reagents except sample or standard. For homogenization, all the test tubes were jerked with the help of vortex for 15 seconds before being kept 30 minutes for proper reaction. By using UV spectrophotometer (set wavelength λ =725nm), absorbance of reaction mixture was recorded against a blank. TTC was demonstrated as (mg of GAE/gm of dry extracts) by using calibration curve of gallic acid.

Analysis of Antioxidant Activity

Qualitative Antioxidant Tests

For qualitative antioxidant assay, thin layer chromatographic (TLC) technique was used [19]. This investigation was conducted based on DPPH's radical scavenging activity. Ascorbic acid and different pulse extracts were mixed with the solvent separately, and then those diluted solutions were spotted by capillary tube on pre-coated TLC plates with silica gel. The development of a chromatogram was done instantaneously by utilizing three distinct elution systems i.e. polar (H_2O: CH_3OH: $CHCl_3$ = 1:10:40), medium polar (CH_3OH: $CHCl_3$ = 1:5) and non-polar (n-hexane: acetone = 3:1). Mobile phase was allowed to migrate up to the specified line. The TLC plates were then allowed to spontaneously evaporate once the chromatogram was generated. After that, the TLC plates were inspected under both short (254 nm) and long (360 nm) wavelength UV light to examine for the presence of fluorescent and UV positive substances. Then, 2,2-Diphenyl-1-picrylhydrazyl solution (0.02% w/v) was spread over the chromatogram developed TLC plates for producing yellow or pale-yellow color spots to confirm the existence of antioxidant compounds.

Quantitative Antioxidant Analysis

DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging assay:

The quantitative antioxidant capacity was tested using DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging assay method with minor modifications [20]. Different concentrations of sample extracts (4000-15.6 µg/mL) and ascorbic acid (1000-3.9 µg/mL) were made by serial dilution. 10µL each sample extract concentration and 190µL of DPPH solution in methanol were poured into each microwell plate and kept at normal temperature (25^0C) in absence of light for 30 minutes. By using Thermo Scientific Multiskan Ex microplate photometer (set wavelength λ =517nm), absorbance of reaction mixture was recorded against a blank. The log concentration vs. percentage of scavenging activity calibration curve was constructed from the reading of absorbance. Scavenging activity denoted as SC_{50} (required concentration which scavenges free radical 50%).

Antibacterial Activity Test

In vitro antibacterial activities of pulse extracts against pathogenic bacteria (*Staphylococcus aureus and Escherichia coli)* were observed by the Kirbey agar disc diffusion method [21]. According to the method, 10 µL test extract solution from 250 µg/10 µL and 500 µg/10 µL prepared solutions were applied to the discs aseptically getting concentrations of 250 µg and 500 µg per disc, respectively. Ciprofloxacin (30 µg/disc) was applied as standard to compare zone of inhibition produced by extract against the test organisms. Finally, extract containing discs were placed in nutrient agar medium seeded with the test organisms containing petri dishes. The zone of inhibition was evaluated after incubating the petri dishes at 37^0C for 24 hours.

RESULTS AND DISCUSSION

Phytochemicals are non-nutritional bioactive molecules exist in plant parts such as leaves, barks, flowers, fruits and roots. Since ancient times, researchers have studied natural plants in desire of new medicinal products. Consequently, a diverse array of medicinal plants possessing therapeutic properties is being utilized in the treatment of various pathogenic diseases [22].

In this study, investigation of phytochemicals from crude methanolic extracts revealed the existence of biologically active micro-molecules (phenols, flavonoids and tannins) inside the pulses (Table 2). These secondary metabolites are responsible for strong antioxidant activity. Phenolic compounds are generated from phenylalanine and tyrosine, which exist ubiquitously in plants [23]. Plant phenolic compounds are also highly significant because they possess hydroxyl groups, which enable them to act as scavengers. Plant with rich in phenolics delays lipid peroxidation and enhances the nutritional content and quality of food [24]. Among the different varieties of pulses, BARI Faba Bean showed a significant presence of phenols (271.63mg GAE/g). Plants naturally produce flavonoids that may be beneficial to human health. Research on derivatives of flavonoids has demonstrated a broad spectrum of antimicrobial, antiviral, anti-inflammatory, anticancer, and anti-allergic properties [25]. It has been demonstrated that flavonoids are quite efficient at scavenging the most oxidizing molecules (free radicals and singlet oxygen) related with a wide number of disorders [26]. In this study, BARI Mung-6 exhibited highest quantity of flavonoids (233.99mg QE/g). Previous investigations have revealed that tannins are responsible for strong immunomodulatory action [27]. Our findings showed BARI Mung-6 possess remarkable presence of tannins (476.32mg GAE/g**)**.

Bioactive macromolecules with antioxidant activity play a key role in controlling the body's redox status and minimizing the damage caused by diseases or drugs [28]. Antioxidants are chemicals that scavenge or neutralize various forms of free radicals, defending us from disorders linked to them [29]. Numerous antioxidant compounds are abundant in most plants. The majority of antioxidant chemicals, including

polyphenols, flavonoids, and tannins, have important therapeutic benefits. Phenolic chemicals reduce the chance of acquiring serious long-term diseases such as diabetes, hepatic disease, cancer, gout, and stroke. Tannins also lessen DNA mutation and lipid peroxidation [30]. In addition, flavonoids also have been exhibited hepatoprotective, renal, vascular, inflammation and various allergic disorders protection also [31]. In qualitative antioxidant assay, different pulse extracts revealed the presence of UV positive and fluorescent compounds at both 254 nm and 360 nm and formation of yellow spots on pre-coated silica gel TLC plate subsequent spraying solution containing DPPH which confirm the existence of antioxidant compounds. The positive result of qualitative antioxidant test encourages to find outs the quantitative antioxidant activity of those sample extracts. Quantitative antioxidant activity was investigated by using DPPH free radical scavenging assay. Common stable free radical DPPH is easily protonated by antioxidant compounds, forming the stable molecule DPPH-H, which lead to conversion of color from violet to light yellow [32]. In order to unstable in nature, DPPH targets adjacent cells in our body swiftly. As a result, it induces lipid peroxidation, sulfhydryl group stimulation, and DNA base disruption. In long period of time, it eventually leads to the development of potentially fatal medical disorders. The antioxidant compounds found in various plants assist us in preventing the cell damage caused by different kinds of oxidative stress. In the DPPH free radical scavenging assay, both the sample extracts and standard (ascorbic acid) scavenged the DPPH varying magnitude based on their concentration [33] and the standard ascorbic acid showed SC_{50} of 16.61 µg/mL. Among different varieties of pulses, BARI Mash-3, Chola-10, Faba Bean and Motor-3 exhibited strong scavenging activity (Table 3 and Figure 2).

Moreover, the antibacterial activity of tested pulse crude sample was examined against gram positive and gram-negative bacteria namely *Staphylococcus aureus* and *Escherichia coli* using Kirby disc diffusion method. In this study, compared with the standard ciprofloxacin among all varieties of pulse extract BARI Chola-10 and BARI Mash-3 exhibited the high zone of inhibition (13mm) against *Staphylococcus aureus* and *Escherichia coli* respectively and BARI Mash-3 and BARI Motor-3 showed medium whereas BARI Felon-1 and BARI Chola-5 showed low zone of inhibition (Table 4). The existence of flavonoid, tannin, and alkaloid in the crude sample extract may account for its antibacterial action. Flavonoid's antibacterial action was demonstrated by completing with bacterial cell walls or binding to adhesions [34]. Tannins showed antibacterial action via binding to proteins, blocking enzymes, or damaging bacterial cell membranes. Therefore, the present study revealed that the different varieties of pulses recently developed by BARI possess significant antioxidant and antibacterial activity owing to presence of high concentration of total phenols, flavonoids and tannins.

Table 2: Total phenolic, flavonoid, and tannin contents of different varieties of pulses

Varieties of Pulses	TPC (mg GAE/g)	TFC (mg QE/g)	TTC (mg GAE/g)
BARI Masur-3 (Lentil)	25.4	87	360
BARI Masur-8 (Lentil)	24.0	96	44
BARI Mung-6 (Mung bean)	5.21	233.99	476.32
BARI Mung-8 (Mung bean)	5.74	192.03	395.17
BARI Chola 5 (Chickpea)	52.31	159.04	80.26
BARI Chola 10 (Chickpea)	124.92	170.63	152.48
BARI Khesari-2 (Grass pea)	156	206	211
BARI Khesari-3 (Grass pea)	149.8	186	218.2
BARI Mash-3 (Black Gram)	154	72.53	90.35
BARI Mash-4 (Black Gram)	136.4	68.16	71.71
BARI Arhar	213.5	162.20	37.82
BARI Faba beans	271.63	201.9	305
BARI Felon-1 (Cowpea)	70.18	203.3	154.81
BARI Motor-2 (Field Pea)	91	42	68
BARI Motor-3 (Field Pea)	134	77	132

Table 3: SC_{50} values of different varieties of pulses

Varieties of Pulses	SC_{50} values (µg/mL)
BARI Masur-3 (Lentil)	263.02
BARI Masur-8 (Lentil)	97.27
BARI Mung-6 (Mung bean)	225.42
BARI Mung-8 (Mung bean)	244.91
BARI Chola 5 (Chickpea)	120.77
BARI Chola 10 (Chickpea)	76.48
BARI Khesari-2 (Grass pea)	88.51
BARI Khesari-3 (Grass pea)	108.14
BARI Mash-3 (Black Gram)	74.65
BARI Mash-4 (Black Gram)	120.23
BARI Arhar	175.38
BARI Faba beans	95.50

Table 4: Zone of inhibition of different varieties of Pulse extracts

Different Varieties of Pulses		Zone of inhibition in diameter (mm) Against bacterial strains	
Sample extracts	Dose (µg/disc)	*Staphylococcus aureus*	*E. coli*
BARI Masur-8 (Lentil)	250	8	0
	500	11	9
BARI Mung-6 (Mung bean)	250	8	0
	500	10	8
BARI Mung-8 (Mung bean)	750	0	10
	1000	0	12
BARI Chola-5 (Chickpea)	750	8	0
	1000	11	8
BARI Chola-10 (Chickpea)	250	8	0
	500	13	9
BARI Mash-3 (Black Gram)	250	9	10
	500	11	13
BARI Mash-4 (Black Gram)	250	5	5
	500	8	7
BARI Arhar	250	7	0
	500	12	8
BARI Felon-1 (Cowpea)	750	8	0
	1000	10	8
BARI motor-3 (Field Pea)	250	8	8
	500	10	11
Ciprofloxacin (Std)	30	29	30

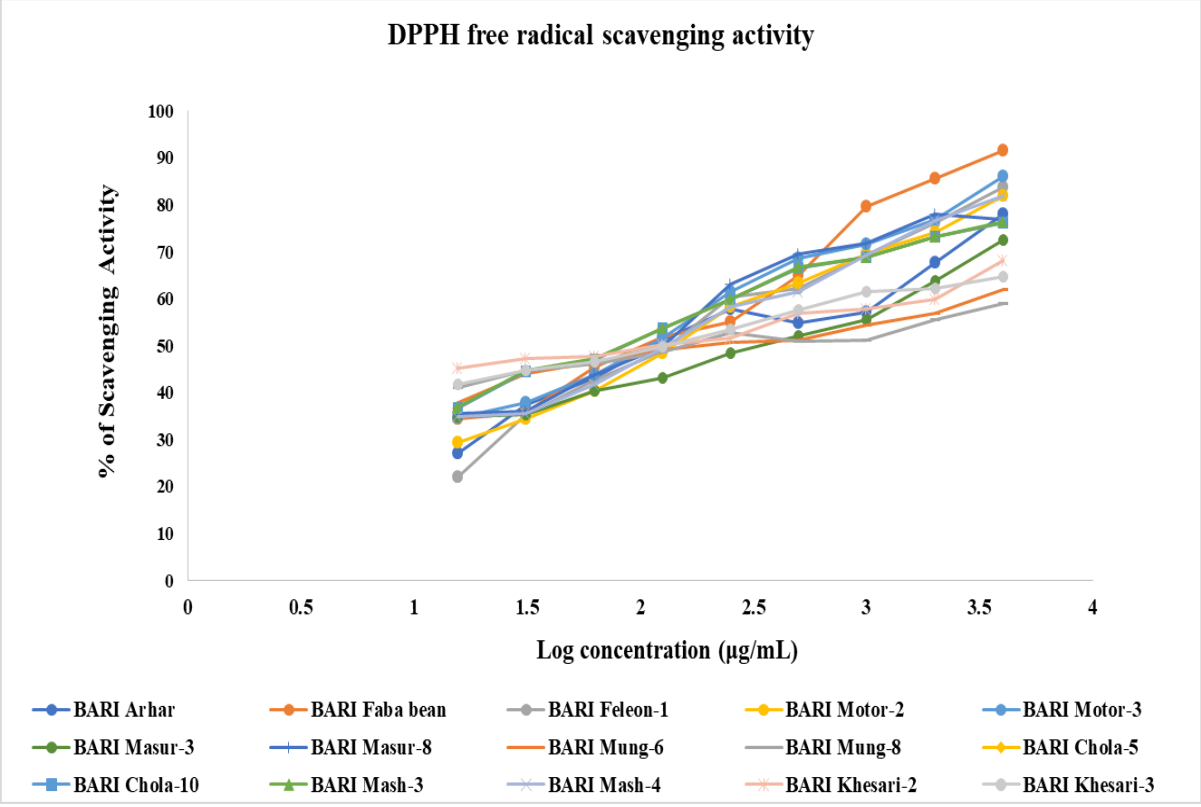

Figure 2: Graphical presentation of DPPH free radical scavenging activity of different varieties of pulse extract

CONCLUSION

Our study was performed on the different varieties of pulses recently developed by Pulses Research Centre of Bangladesh Agricultural Research Institute (BARI), Ishwardi, Pabna, Bangladesh to investigate antioxidant and antibacterial activities linked to its particular use in traditional medication. Experimental findings revealed that the varieties of pulse extracts are rich in various phytochemicals (phenols, flavonoids and tannins) which have very good free radical scavenging capacity and also antibacterial activity. This scientific study will enhance the consumption of pulses as nutritional supplements and help to identify the exact mechanism and isolate the bioactive compounds for further pharmacological applications.

Conflict of interest

There is no conflict of interest.

Financial Support

None declared.

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