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Biochemical analysis and evaluation of free radical scavenging activity of bitter gourd seeds' aqueous and ethanolic extracts: Comparative study

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Abstract
Over the last few decades, seed research has shown significant enthusiasm, particularly in the food and herbal medicine sectors. But the majority of the time, the seeds of the vegetables are discarded as food waste, without knowing much about their potential as a source of bioactive compounds. Among the different medicinal plants, *Momordica charantia* Linn., belonging to the family Cucurbitaceae, natively known as Bitter gourd, has many biologically active chemical compounds. Therefore, our present study aims at the comparative phytochemical investigation with the analysis of antioxidant efficacy, nutrient composition, and proximate parameters of bitter gourd seed's aqueous and ethanolic extracts. The results revealed that the ethanolic extract of bitter gourd seeds shows the highest amount of extractive value (66.22±2.78%) as well as total polyphenol (47.14±0.07 mg GAE/g of DT)), flavonoid (16.83±0.04 mg QE/g of DT), and tannin content (23.24±0.24 mg TA/g of DT). On the contrary, an aqueous extract shows the maximum amount of carbohydrate (68.56±0.09 mg GL/g of DT), along with in vitro antioxidant potential, i.e., inhibition percentage for DPPH (97.74±0.06%), for ABTS (98.09±0.16%). Depending on the overall results, including the proximate parameters, it can be stated that the seeds of bitter gourd can be utilized as a persuasive source of phytochemicals that can treat many oxidative stress-related disorders and may have some beneficial roles in pharmaceuticals as well.

Keywords: *Momordica charantia*, Seeds, phytochemicals, antioxidants, nutritional elements, proximate parameters

Introduction
From time immemorial, various plants have been utilized with the perspective of both as medicine and vegetable [1]. Vegetables are considered one of the significant sources of nutrients, minerals, dietary fiber, antioxidants, and other beneficial phytochemicals [2]. The combinations of therapeutic and vegetable usage have made some plants very popular over the years. In present days, approximately 80% of the world's population depends on the herbal system of medicine. Thus nutrients obtained from plants have emerged as new and potential means of health aid and preventing and treating different diseases [3]. *Momordica charantia* Linn. (Bengali name Karela), a therapeutic vine belonging to the family Cucurbitaceae, commonly known as Bitter melon or Bitter gourd, is widely distributed in Bangladesh, Malaysia, China, India, and tropical Africa. Bitter gourd contains many biologically active chemical compounds, including proteins, triterpenes, steroids, saponins, alkaloids, and flavonoids [4-6].
Clinical studies with multiple controls have confirmed the benefit of bitter melon in cade of treatment for diabetes. Alpha and beta momarcharin are two proteins predominantly found in bitter melon, proven to have a strong inhibitory effect against the AIDS virus. In addition, this plant has been reported to have other biological and pharmacological activities, including anti-bacterial, anti-fungal, anti-viral, anti-parasitic, anti-fertility, anti-tumor, and anti-carcinogenic properties [7-9]. Bitter gourd fruits are mainly used as food items. These fruits come in various shapes and sizes [10-12]. The bitter melon that is more common in India has a narrower body, pointy ends, and a surface covered in spiky, triangular "teeth" and ridges, and their color is either green or greenish-white. These little fruits are popular in Southeast Asia and India. The pods are smaller and vivid orange, with delicious red seeds when ripe. Recently, bitter gourd seeds came into the limelight for their immense nutritional value and medicinal properties [13-16].

It is found that seeds also harbor several essential phytochemicals that include the Urease enzyme and some important amino acids – valine, threonine, methionine, isoleucine, leucine, phenylalanine, and glutamic acid [17, 18].
Despite having such bioactive compounds, the seeds are thrown away most of the time, ultimately generating biowastes. However, in recent times, many scientific investigations have reported the potential of those unused parts because of the source of essential phytochemicals like polyphenols, flavonoids, alkaloids, sugars, vitamins, and minerals [19, 20].
Based on the above scientific statements, our present study aims to compare phytochemical screening with subsequent analysis of the antioxidant, nutrient, and proximate activity of bitter gourd seeds extract. The overall workflow is being represented through a graphical abstract in Figure 1.

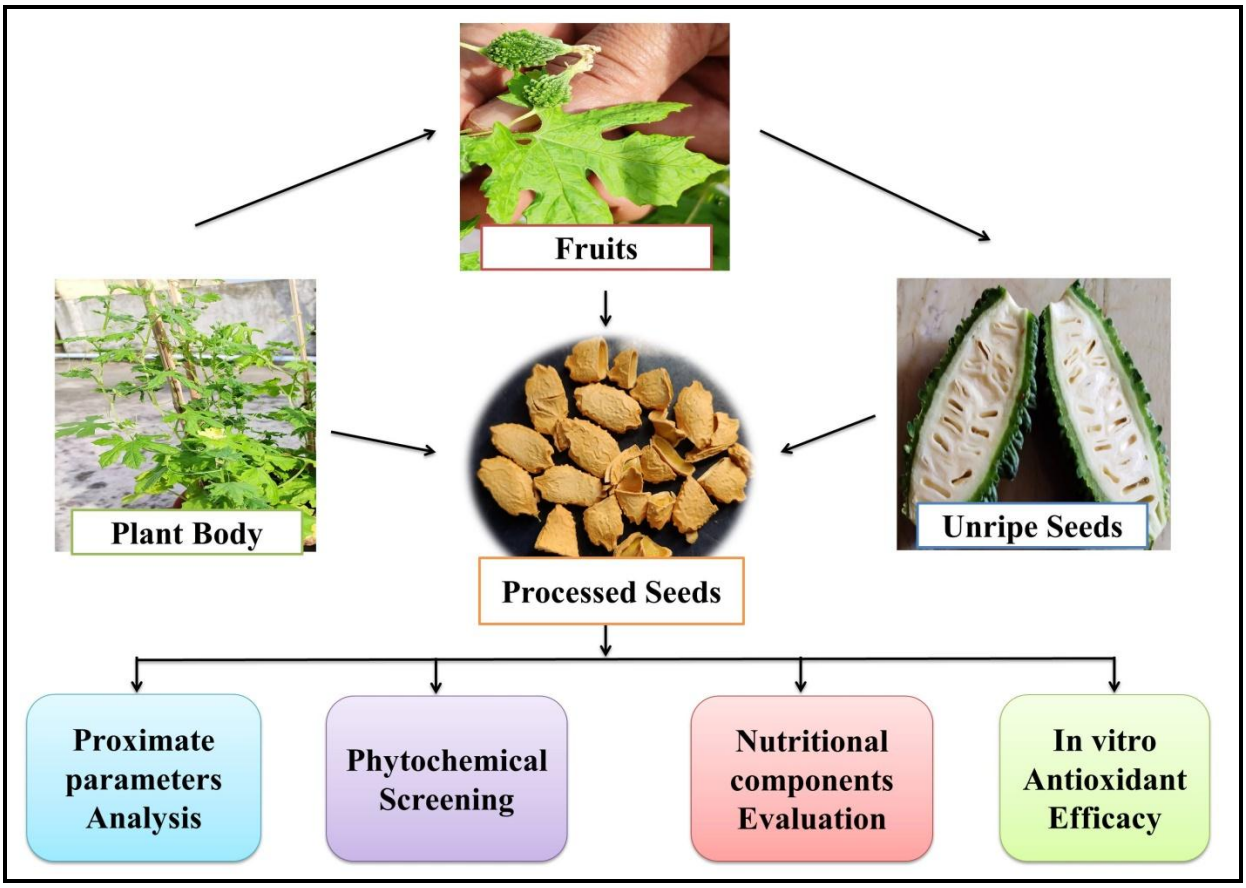


Fig 1: The Graphical Presentation of the Work Flow

Materials and Methods

Chemicals and reagents

All the chemicals and reagents used in the experiments were of analytical (AR) grade and purchased from Institutional enrolled chemical suppliers. PC Based Double Beam Systronics spectrophotometer 2206 was used for quantitative assays to determine the specific absorbance.

Sample collection and preparation

The seed samples of bitter gourd (Figure 2) were collected from the North 24 Parganas, West Bengal, in November-December 2021. After collecting the samples, the seeds were carefully separated, cleaned, washed well to remove all the impurities, and are shade dried separately until all the water molecules were evaporated; next, samples were mechanically ground and coarsely powdered. Finally, the powder was subjected to solvent extraction with double distilled water and 100% ethanol for their respective purposes.

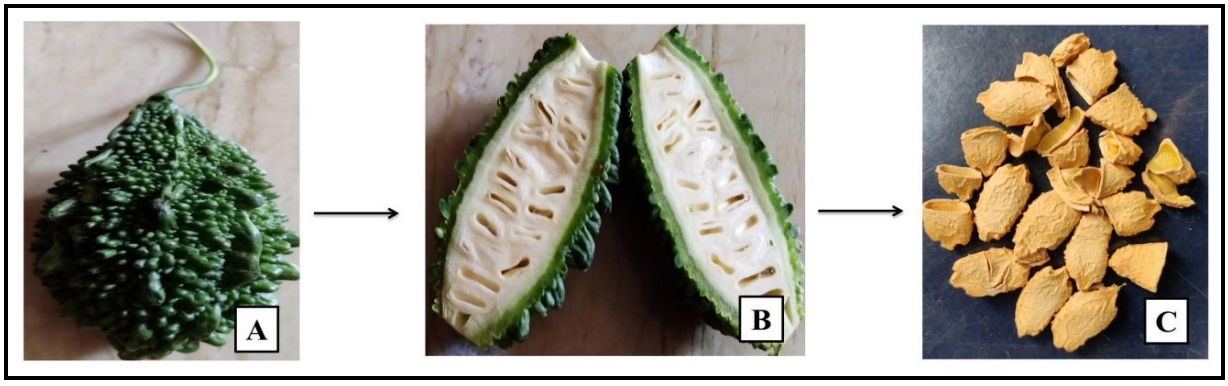


Fig 2: The Steps in Sample Collection [A: The Bitter Gourd Fruit; B: The Unripe Seeds inside the Fruit; C: The Dried Seeds]

Extraction technique
50 ml of solvent (distilled water and 100% ethanol) in a conical flask was added to 1gm of seed powder. They were kept in a shaker for 24 hours at room temperature. All the extracts were filtered using filter paper (Whatman No.1). The filtered solution of the extracts was stored at 4°C and diluted for further studies according to the need for the specific assay.

Proximate Analysis
Extractive value
The extractive value was determined by the standard method [21] with slight alteration using both the distilled water and 100% ethanol as solvents. The percentage extractive value was calculated using the following formula:
Extractive value (%) = Weight of dried extract/ Weight of plant material × 100

Determination of Total Moisture Content
The standard method was used to evaluate the total moisture content (MC) of the seeds [22]. From the following formula, the total moisture content of the seeds was calculated:
MC = WI - WF / WI × 100
[Where MC = Moisture Content, WI = Initial weight of Samples, WF = Final weight of samples]

Determination of pH of the Leaves Extracts
To determine the pH of both aqueous and ethanolic extracts of the seeds, the standard method of Aremu *et al*., 2010 was used with proper adjustments and modifications [23].

Determination of Conductivity of the Extracts
1 g of the fresh seeds was pestle in 10 ml double distilled water. This was filtered, and the conductivity of the seed extract was determined after calibrating the conductivity meter with standard buffer solutions (pH 4, pH 7, and pH 10) and double distilled water at the 25 °C temperature [24].

Quantitative Phytochemical Assays:
Quantification of Total Phenolic Content (TPC)
The total polyphenol contents were determined by using the Folin-Ciocalteu method with adjustments. Gallic acid was used for the preparation of the standard curve. The absorbance was read at 765 nm. The results were expressed as mg Gallic acid equivalents/g of dry tissue [25].

Quantification of Total Flavonoid Content (TFC)
Aluminium chloride colorimetric assay with adjustments was used for the determination of flavonoids. The absorbance was read at 510 nm. Quercetin was used for the preparation of the standard curve. The results were expressed as mg Quercetin equivalents/g of dry tissue [26].

Quantification of Total Tannin Content (TTC)
The Folin-Ciocalteu colorimetric assay with adjustments was used to determine total tannins. The absorbance was read at 700 nm. Tannic acid was used for the preparation of the standard curve. The results were expressed as mg Tannic acid equivalents/g of dry tissue [27].

Nutritional Quantification
Quantitative Screening of Carbohydrate
The standard Anthrone method was used with slight modifications to estimate total carbohydrate content in various seed extracts [28]. Absorbance was read at 620 nm. Glucose was used as a standard. The polysaccharides content was expressed as mg Glucose Equivalent/g dry weight.

Quantitative Screening of Protein
The total protein content was measured by the conventional Lowry's method (absorbance at 660 nm.) [29]. Bovine serum albumin was used as a standard reagent. Whole seed samples were extracted with phosphate buffer (pH 7.4). The samples and reagents were used in a 1:1 ratio. Total protein content was expressed as mg Bovine Serum Albumin Equivalent/g fresh weight.

Quantitative Screening of lipid
The total lipid content was estimated using the standard method [30]. Then, the total lipids were extracted with chloroform: methanol: water (2:2:1.8 ratio) mixture (10 ml mixture per 1g sample) by cold extraction method (Bligh, E.G., *et al*., 1959). Finally, the total lipid content (%) of the sample is calculated by using the following formula:
Total Lipid Contents (%) = (lipid weight in aliquot × chloroform layer volume)/aliquot volume.

Determination of Antioxidant Activity
DPPH Radical Scavenging Assay
2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was evaluated using the standard protocol with slight adjustments [31]. The standard curve was made using ascorbic acid. Absorbance was read at 517 nm. The DPPH radical scavenging capacity was expressed in terms of Ascorbic Acid Equivalent, as the percentage of inhibition of the assay was calculated by the following formula:
% Inhibition of DPPH= OD of control-OD of sample/ OD of control*100

ABTS Free Radical Scavenging Capacity Assay
The radical scavenging activity of ABTS (2, 2-azinobis- 3-ethylbenzthiazoline-6-sulphonic acid) was evaluated using the standard protocol with minor adjustments [32]. The standard curve was made using ascorbic acid. Absorbance was read at 734 nm. The DPPH radical scavenging capacity was expressed in terms of Ascorbic Acid Equivalent, as the percentage of inhibition of the assay was calculated by the following formula:
% Inhibition of DPPH= OD of control-OD of sample/ OD of control*100

Statistical Analysis
All the experimental measurements were triplicate and expressed as the average ± standard deviations. The magnitude of the means, standard curve, standard errors, standard deviations, one-way ANOVA, and paired *t*-test were calculated using Microsoft Excel 2010 Software. $P<0.05$ is accepted as statistically significant.

Results and Discussions
Proximate Analysis
The proximate parameters were analyzed using definite procedures and represented in Table 1 and Figure 3. The current research revealed that the extractive value of ethanolic extract of bitter gourd seeds is higher (66.22±2.78%) than the aqueous extract (56±1.33%). The overall moisture content for the sources is determined to be 7.71±0.13%. The pH values of ethanolic and aqueous extracts are found to be 7.07±0.02 and 5.21±0.02, respectively. In the case of both the extractive value and pH determination, statistical analysis was done based on which p-value obtained is less than 0.05, which

demonstrated statistically significant differences in those values between the two type types of extracts. The value for the conductivity of aqueous extract is estimated to be 5.35±0.017 mS/cm.

Table 1: Results of Proximate Parameters

Sample	Moisture content%	Conductivity (mS/cm)
Bitter gourd seeds	7.71±0.13	5.35±0.017

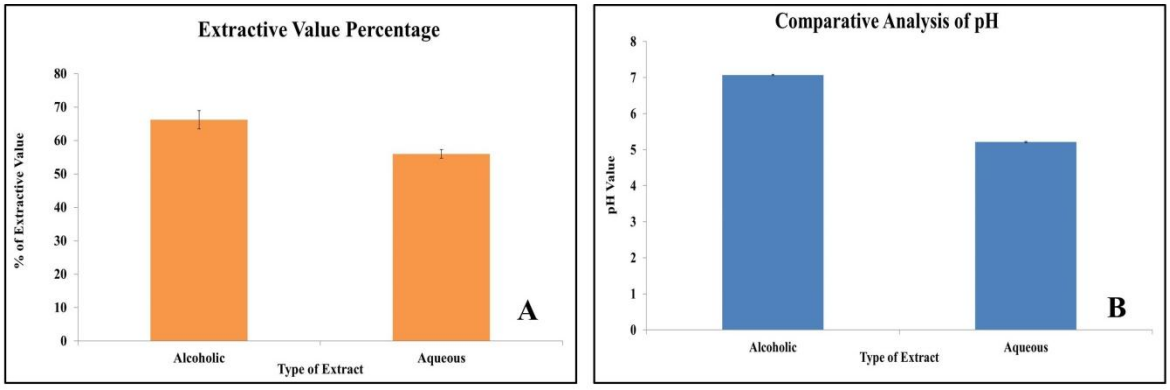


Fig 3: Results Showing Proximate Analysis [A: Extractive Value (%); B: pH Value]

Quantitative Phytochemical Assays

The present study aims to determine the phytochemical investigation and in vitro antioxidant efficacy of bitter gourd seed aqueous and ethanolic extracts. Since it is well established that the antioxidant property of plants largely depends on the total polyphenols (TPC) and flavonoids (TFC) content as well as total tannin content (TTC), we quantify their concentration using standard protocols.

The total polyphenol content (TPC) of both extracts of bitter gourd seeds was estimated. The result showed that bitter gourd ethanolic extract contained the highest amount of polyphenol content, i.e., 47.14±0.07 mg GAE/g of dry tissue, wherein the case of aqueous extract, it was found to be 36.68±0.07 mg GAE/g of dry tissue (Figure 4A). After performing one-way ANOVA followed by paired *t*-test, the obtained p-value< 0.05 stated that the difference between the amount of polyphenols in both extracts is statistically significant. Polyphenol compounds are reactive species towards oxidation and regulate bio-physiological activity.

The total flavonoid content (TFC) of both the extracts of bitter gourd seeds was determined. The maximum amount of flavonoid content was present in bitter gourd ethanolic extract, i.e., 16.82±0.04 mg QE/g of dry tissue, and the lowest amount, i.e., 8.48±0.17 mg QE/g of dry tissue, present in aqueous extract (Figure 4B). The obtained p-value <0.05 via one-way ANOVA followed by paired *t* test, which exhibited the statistical significant difference between the presences of flavonoids in both types of extracts. Depending on their specific structure, flavonoids compounds can inhibit all possible reactive oxygen species.

The total tannin content (TTC) estimation of both extracts of bitter gourd seeds revealed that tannin content was higher in ethanolic extract, i.e., 23.24±0.24 mg TA/g of dry tissue, in comparison to aqueous extract i.e., 15.85±0.47 mg TA/g of dry tissue (Figure 4C). After statistical analysis of the data via one-way ANOVA followed by paired *t*-test, the resulting p-value <0.05 declared the statistically significant difference in total tannin content in both types of extracts of bitter gourd seeds.

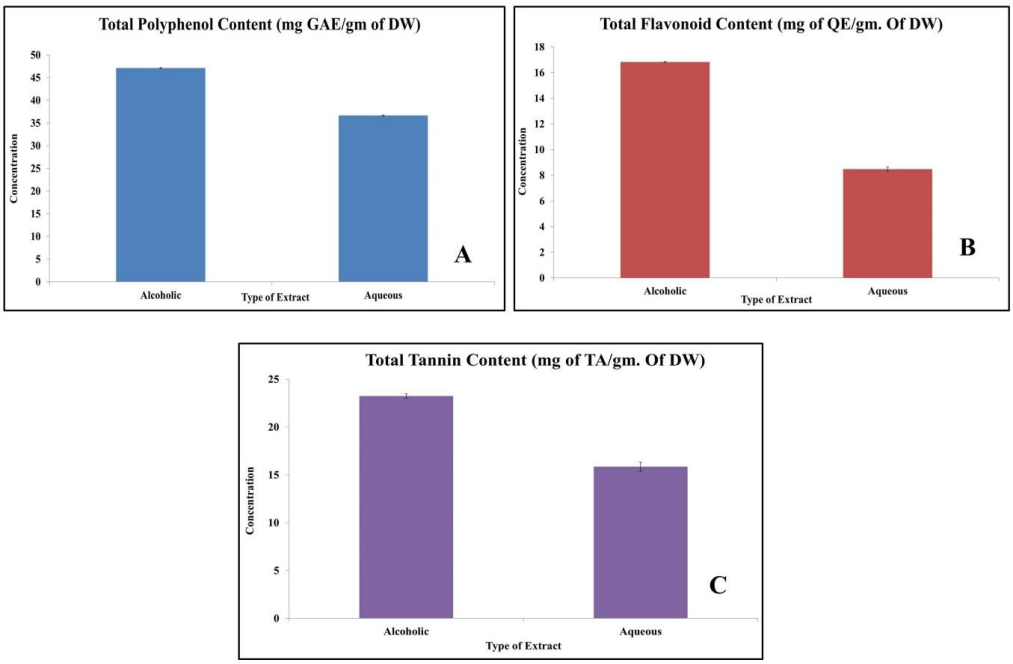


Fig 4: Results Showing Phytochemical Screening [A: Total Polyphenol Content (mg GAE/G OF DW); B: Total Flavonoid Content (mg QE/g of DW); C: Total Tannin Content (mg TA/g of DW)]

Nutritional Quantification

In addition of estimating proximate and phyto-component analysis, various nutritional parameters were also evaluated and represented through Table 2 and Figure 5. The total carbohydrate content for each type of extracts had been determined and it was observed that aqueous extract of bitter gourd seeds possessed more amounts of carbohydrates when compared to ethanolic extract and the values are 68.56±0.09 mg GL/g of dry tissue and 29.71±0.27 mg GL/g of dry tissue respectively (Figure 5). The data obtained via this biochemical assay undergone through statistical data analysis procedure. Results showed that the difference in carbohydrates in both types of seed extracts is statistically significant ($p < 0.05$).

The amount of total protein content was found to be 54.55±0.20 mg BSA/ gm. of DW, whereas the total lipid content was determined to be 6±2%.

Table 2: Results of Nutritional Quantification

Sample	Total Protein Content (mg BSA/ gm. Of DW)	Total Lipid Content (%)
Bitter gourd seeds	54.55±0.20	6±2

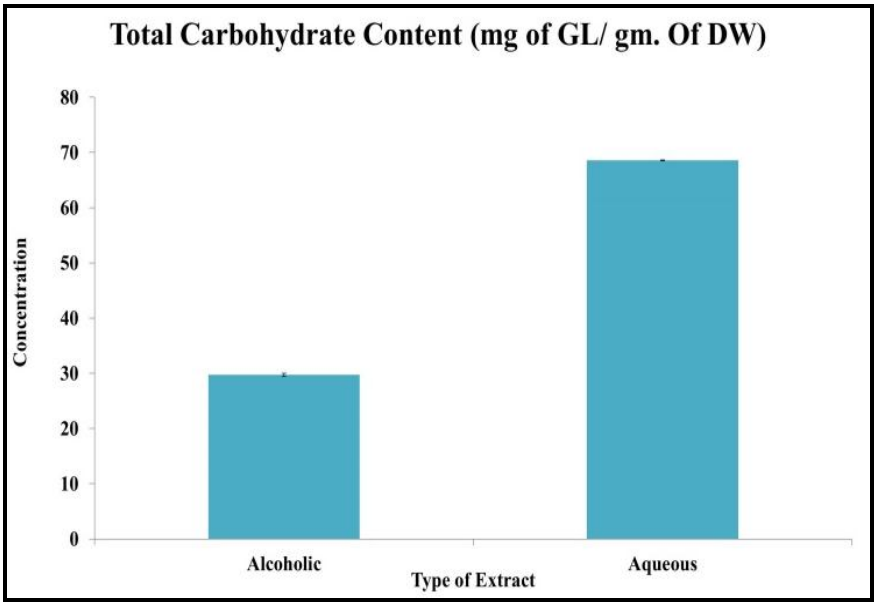


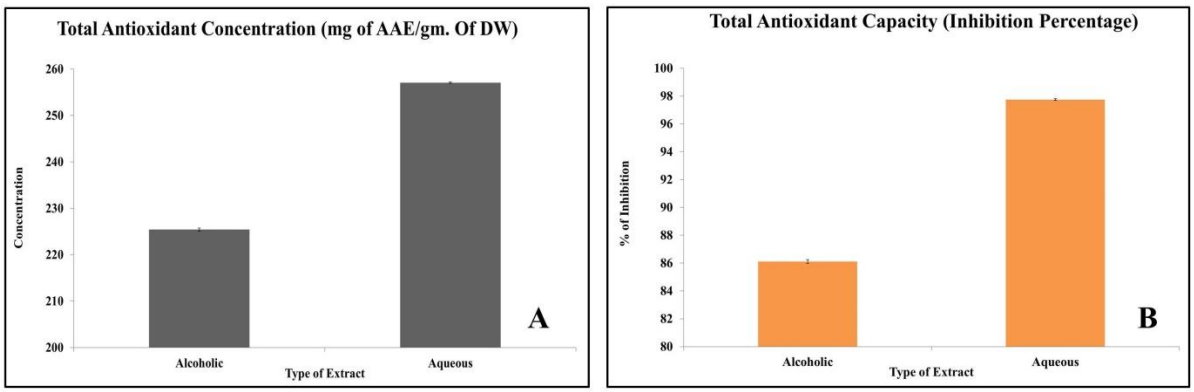
Fig 5: Results Showing Nutritional Quantification for Total Carbohydrate Content (mg of GL/gm. Of DW)

Determination of Antioxidant Activity

Bioactive compounds can act as an antioxidant by scavenging the free radicals. Both DPPH and ABTS are stable free radicals used to investigate the free radical scavenging capacity of an antioxidant. The inhibition percentage of DPPH radical scavenging assay was found to be the highest in bitter gourd aqueous extract, i.e. 97.74±0.06%, on the other hand, ethanolic extract showed the lowest inhibition percentage, i.e.86.12±0.12% (Figure 6A and 6B). Corresponding total antioxidant concentration was also determined.

Similarly, the inhibition percentage of ABTS radical scavenging assay was the highest in bitter gourd aqueous extract, i.e. 98.09±0.16%. On the other hand, ethanolic extract showed the lowest inhibition percentage, i.e.87.38±0.08% (Figure 6C and 6D). Corresponding total antioxidant concentration was also estimated.

In both DPPH and ABTS assays, statistical analysis of the respective data was performed. As a result, the obtained p value is less than 0.05 in both cases, which in turn display statistically significant differences in free radicals inhibition property between both the extracts.



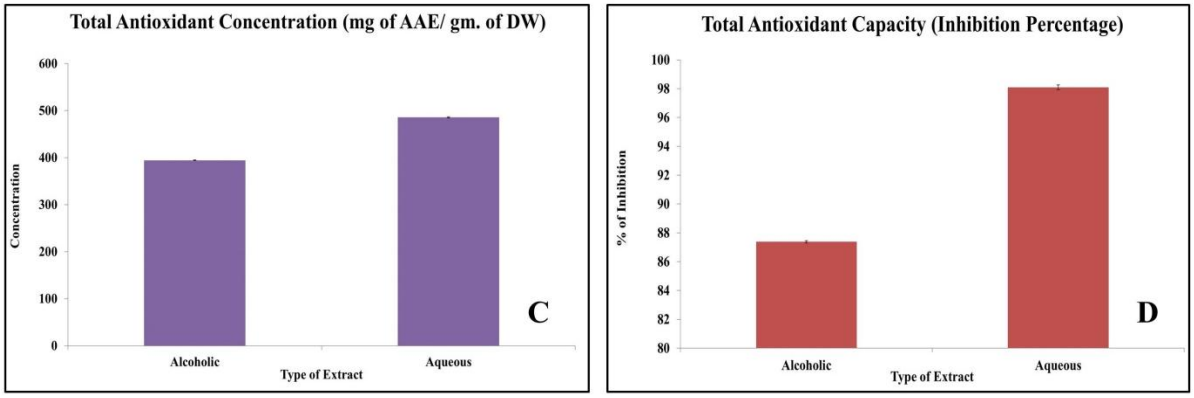


Fig 6: Results Showing Antioxidant Activity [A: Total Antioxidant Concentration (mg AAE/gm. of DW) For DPPH; B: Inhibition Percentage for DPPH; C: Total Antioxidant Concentration (mg AAE/gm. of DW) for ABTS; B: Inhibition Percentage for ABTS]

Conclusion

Overall, most scientific studies suggest that regular consumption of bitter gourd indeed can circumvent various health-related problems either by its prophylactic or therapeutic actions. The curative nature of seeds is perhaps due to the presence of different secondary metabolites such as polyphenols, flavonoids, tannins, and many other phytonutrients such as carbohydrates, proteins, and lipids are rich in showing antioxidant activity [33].The successive extraction of seeds of both bitter gourd in double distilled water and 100% ethanol solvent supports the preliminary phytochemical screening that may be helpful in detecting bioactive principles and subsequently lead to drug discovery and development [34]. On the other hand, quantitative analysis showed the presence of polyphenols and flavonoids that enhanced their higher antioxidant activity as evident from the free radical scavenging assay. The study's findings support the fact that seeds as a potential part of traditionally used medicinal plants are the primary source of therapeutically used antioxidants [35]. Based on the results obtained, it can be concluded that bitter gourd seeds might be *a* rich source of natural antioxidants and various phenolic and flavonoid compounds that increase their nutritional and medicinal aspects [36-38].

Conflict of Interest: The authors declare that there are no conflicts of interest regarding the publication of this research article.

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