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Investigation of Nutraceutical Potential, *in vitro* Antioxidant and Free Radical Scavenging Activity of Indian Royal Jelly

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ABSTRACT: *Apis mellifera*, a priceless bee species is known to produce various nutritional products. Royal Jelly (RJ) is one such bee product which has high nutritive value, functional and biological qualities. However factors such as bee species, environment, season, collection technique and larval age affect the composition of RJ at macro and micro levels thus there is a need to evaluate Indian Royal Jelly (IRJ) for its chemical properties. So in the present work IRJ samples collected from Southern, Central and Northern regions of India were evaluated for physicochemical parameters (nature, color, appearance, odor, and solubility,) residual content (moisture content, ash value and pesticide content), chemical attributes (total polyphenol content, total flavonoid content,), nutraceutical potential (total fat, protein, carbohydrate content, and energy), and antioxidant activity (DPPH assay, ABTS assay and reducing power,). The total flavonoid content and total phenolic content were in the range of 0.119-0.321 mg quercetin/g of IRJ and 25.2844-68.203 mg Gallic acid/g, respectively. High energy, protein and low fat value suggested IRJ as a suitable nutraceutical agent. Antioxidant activity (IC_{50}) of IRJ samples was found to be in the order of IRJ II > IRJ IV > IRJ I > IRJ III. Overall, it was observed that the IRJ-II showed better nutritional efficacy, polyphenolic content, and antioxidant properties.

KEYWORDS: Indian Royal Jelly; physicochemical; nutraceuticals; phenolic; flavonoid; antioxidant

1. INTRODUCTION

In stressful environment and unhealthy diet, the free radicals formation in the cells is found increased [1]. It is observed that oxidation of free radicals produce oxidative stress resulting in enzyme deactivation, lipid peroxidation, and aging [2]. Thus, there is tremendous demand for natural products with antioxidant activity. There are several health-promoting bioactive chemicals produced by *Apis mellifera*, the bee species, making them a priceless natural resource. Bee bread, venom, bee pollen and royal jelly (RJ) are some of the high valued bee products [3]. One of the most significant things honeybees generate is RJ, which is regarded to be a naturally occurring chemical that has a high nutritive value with functional and biological qualities. The hypopharingeal glands of worker bees produce this substance, which is utilized to feed both the queen bee and the colony's larvae [4]. Royal Jelly contains all of the nutrients that larvae need to grow and thrive. Drone larvae and worker larvae are fed RJ until the maturation phase. After the maturation phase, worker larvae are fed a combination of RJ, pollen and honey, but the queen honeybee is fed RJ throughout her life cycle. Due to its thick consistency and the presence of undissolved granules of various sizes that have not entirely dissolved, it is often not homogenous. It has a solubility index of 1.1 gram per milliliter (g/mL).

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Depending on how long it's been stored, its colour may change from white to a bright yellow and when it comes to flavour, it is acidic yet sweet at the same time [5].

Proteins, carbohydrates, lipids, mineral salts and water are the major components of RJ. RJ is also a source of vitamins (B1, B2, and B6), enzymes, hormones and polyphenols. A wide range of minerals and trace elements with biological activity are also included (such as phosphorus and sulphate), as well as sodium, potassium, magnesium, calcium, zinc, iron, copper, and manganese [6]. There are a number of biological and therapeutic activities that RJ has been known to perform for centuries, including anti-aging, anti-fatigue, anti-allergic and immunoregulatory qualities [7]. Lipid fraction is chemically interesting and a major essential component of RJ which contains a significant amount of immune system-boosting, unique fatty acid is 10-hydroxy-trans-2-decenoic acid (10-HDA). The 10-HDA content of a high-quality RJ is predicted to be between 1.4 - 1.8 %. This number might fluctuate depending on the location of the RJ's flora and fauna and the harvesting methods used. Structure shows that one of the hydrogens attached to the terminal carbon has been replaced by a hydroxy group, making this an *E*-10-hydroxydec-2-enoic acid. No other natural product contains 10-HDA as high free fatty acid [8].

The amount of 10-HDA detected in Romanian RJ ranged from 0.75 to 3.8 %. The Bulgarian RJ had low fructose levels and high protein and sucrose levels in its chemical composition whereas Romanian RJ shows exactly opposite level of sucrose and fructose. The unique heterogeneity of the royal jelly, is due to climatic changes between the two nations, and even methodologies employed for the same objective may all influence the product's composition. RJ output is also affected by bee races [9]. Fructose and glucose make up the majority of the sugars in RJ. Total sugars often have 90 % fructose and 30 % glucose. RJ is rich in antioxidant content and with probiotic bacterial species it has been shown synergistic effect to produce significant antioxidant activity [10]. Similarly the mineral material composition of RJ, in contrast to honey, is not significantly influenced by anything other than the local geology and vegetation [11]. Therefore factors such as bee diet, bee species, environment, season, collection technique and larval age have an influence on the composition of RJ at macro and micro levels.

Beekeeping being a traditional practice followed in India, is capable of generating huge revenues through export and sale of honey and its related products. RJ and products derived thereof, could benefit the beekeepers in generating additional financial impetus, boosting the economy.

Several pharmacological properties of RJ has been used in foods, cosmetics, and pharmaceutical industries. Previous studies have been conducted on antioxidant activity of RJ [2], [10],[16]. However, there is no study reported for the total polyphenolic content, flavonoid content, nutraceutical potential, and antioxidant activity of Indian Royal Jelly. Thus the aim of present study was to establish the chemical profile of IRJ in terms of total phenols, flavonoids, and proteins and to investigate the nutraceutical potential and antioxidant properties of IRJ from different geographical regions of India.

2. RESULTS

2.1. Physicochemical parameters

All IRJs demonstrated alike physicochemical properties in terms of solubility parameters in water, ethanol, methanol, acetonitrile, and methanol: water, odor (pungent, slightly acidic), Moisture content and Ash value. However, IRJ-I to IRJ-III exhibited thick and viscous nature with milky appearance. Contrasting to all four products, the lyophilized powder was amorphous in nature, and slightly yellowish in appearance (Table 2). The moisture content and ash content of the IRJ IV sample were found to be 3.8 ± 0.30% and 2.59 ± 0.23% respectively. The moisture content of IRJ-I to IRJ-III remained fairly high whereas IRJ-IV, the lyophilized sample showed a very low aqueous content thereby suggesting better shelf-life, microbial stability, and product purity (Table 3).

Table 2. Physicochemical parameters of Indian Royal Jelly.

Test	Observations	
	IRJ-I – IRJ-III	**IRJ-IV**
Nature	Thick, Viscous	Amorphous powder
Color/ appearance	Milky	Slightly Yellowish
Odor	Pungent, Slightly acidic	Pungent, Slightly acidic
Solubility	Water-Partially Soluble	Water-Partially Soluble
	Ethanol-Insoluble	Ethanol-Insoluble
	Methanol-Slightly Soluble	Methanol-Slightly Soluble
	Acetonitrile-Slightly Soluble	Acetonitrile-Slightly Soluble
	Methanol: Water (1:1) - Partially Soluble	Methanol: Water (1:1) - Partially Soluble

Table 3. Comparative data of residual content, polyphenol content, nutritional values of different Indian Royal Jelly samples (N=3)

	Residual content		**Polyphenol Content**	
Sample	**Moisture Content (%)**	**Ash value (%)**	**Total Flavonoids Content (mg QE/g RJ)**	**Total Phenolic Content (mg GA/g of RJ)**
IRJ-I	64 ± 3.12	1.34 ± 0.12	0.129 ± 0.006	25.2844 ± 0.435
IRJ-II	63 ± 2.04	1.2 ± 0.11	0.147± 0.005	25.9906 ± 0.472
IRJ-III	64 ± 2.61	1.4 ± 0.13	0.119± 0.002	24.3429± 0.423
IRJ-IV	3.8 ± 0.30	2.59 ± 0.23	0.321± 0.010	68.2032± 0.934

Nutritional Values

Sample	**Total Sugar (%)**	**Glucose (%)**	**Fructose (%)**	**Sucrose (%)**	**Maltose (%)**
IRJ I	10.49	2.03	4.35	2.21	1.1
IRJ II	10.77	2.09	4.42	2.31	1.3
IRJ III	10.55	2.06	4.25	2.28	1.3
IRJ IV	28.75	5.66	10.54	6.83	3.8

Sample	**Protein (g/100g)**	**Fat (g/100g)**	**Carbohydrates (g/100g)**	**Total Energy(kcal/100g)**
IRJ-I	12.73 ± 1.40	3.1 ± 0.34	18.83 ± 2.07	154.14 ± 23.12
IRJ-II	12.97 ± 1.43	3.2 ± 0.35	18.97 ± 2.09	156.56 ± 23.48
IRJ-III	11.56 ± 1.27	2.96 ± 0.33	17.34 ± 1.91	142.24 ± 21.34
IRJ-IV	34.57 ± 3.80	9.57 ± 1.05	1.38 ± 0.15	229.93 ± 34.49

2.2. Pesticide content

About 166 pesticide were tested including atrazine, azoxystrobin, carbaryl, chlorpyrifos, fenpropathrin, fluvalinate-tau, carbendazim , difenoconazole , dimethomorph, 2,4-DMPF, ımidacloprid, metolachor , profenophos, propamocarb, simazine, thimethoxam, thymol, triadimefon ,trifuralin etc. All pesticides were found to be absent (BDL).

2.3. Chemical evaluation

Table 3 shows TPC and TFC results of the IRJ samples. TPC and TFC was estimated from the calibration curve of GA with the y = 0.2549x + 0.0543 and calibration curve of QE with linear regression equation y = 0.0605x - 0.0568 respectively. The TPC value was expressed in mg equivalent of GA/g while TFC value was expressed in mg equivalent of QE/g of IRJ. The TPC and TFC was found to be higher in IRJ IV which could be attributed to lower moisture content.

2.4. Nutraceutical parameters

Total protein content was estimated using the standard calibration curve of BSA with the linear regression equation y = 0.004x + 0.1152. The protein content and other nutraceutical parameters of IRJ-I to IRJ-III remained uniform in terms of fat, protein, carbohydrate, and total energy. While the lyophilized sample, IRJ-IV showed 3.25-times higher protein level which lead to nearly 1.75-folds higher total energy In addition, a marked decrease (nearly 18-times) in carbohydrate content was observed. The results indicated that the fresh IRJ as well as lyophilized IRJ can be considered as a good nutraceutical product for multiple pharmacological benefits (Table 3).

2.5. Antioxidant activity

2.5.1. *DPPH free radical scavenging activity*

Figure 1a shows the free radical scavenging activity of different IRJ samples and standard AA. Among the IRJ samples, IRJ II showed the highest activity. The scavenging activity of IRJ I,,IRJ II, IRJ III and IRJ IV at a concentration of 100 µg/mL, was 68.41 ± 1.10, 78.67 ± 0.70, 65.55 ± 1.95 and 73.17 ± 0.28 %, respectively, whereas the free radical scavenging activity of AA showed 99.24 ± 0.32 % at the same concentration. The IC_{50} value of IRJ I, IRJ II, IRJ III and IRJ IV was 54.03 ± 0.28, 45.63 ± 0.84, 58.86 ± 1.26 and 47.10 ± 1.14 µg/mL, respectively, whereas the IC_{50} value of AA was 23.8 ± 0.85 µg/mL. (Fig. 1b). The DPPH scavenging activity of different IRJ Samples and AA was followed the order: AA > IRJ II > IRJ IV > IRJ I > IRJ III.

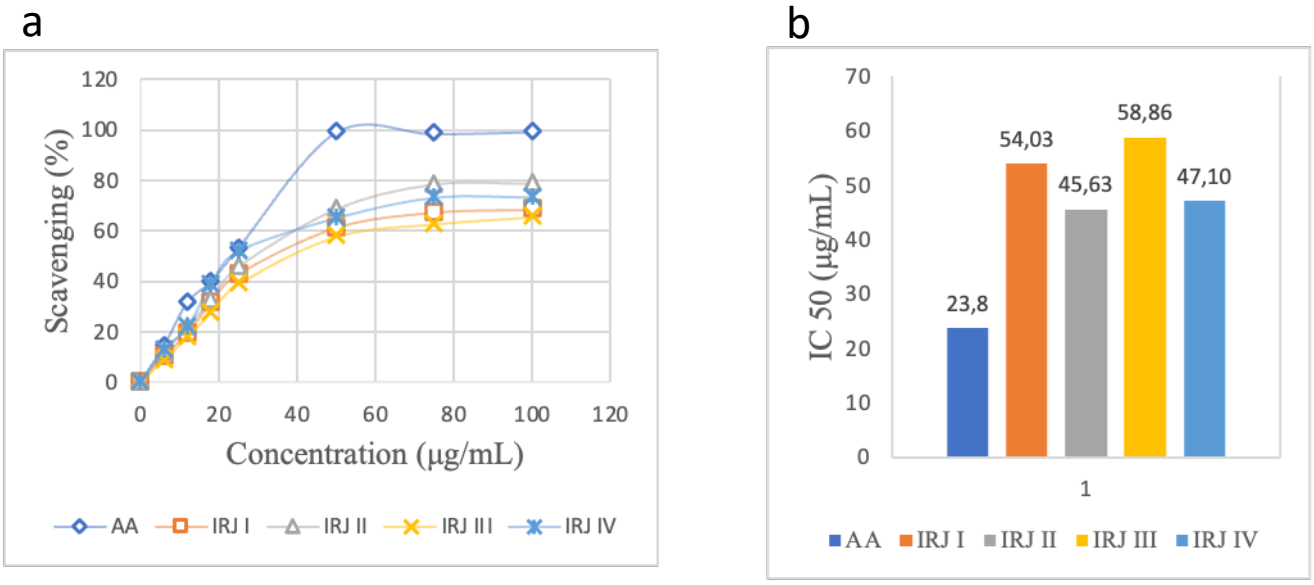

Figure 1. Determination of **a** DPPH inhibition activity and **b** IC50 of IRJ samples (I, II, III and IV). Data were expressed as a mean ± SD (n = 3, $p < 0.05$) for all samples.

2.5.2. *ABTS scavenging assay*

In this case of ABTS assay, the scavenging potential of various IRJ samples and standard AA was compared. The scavenging activity at a concentration of 100 µg/mL of IRJ I, IRJ II, IRJ III and IRJ IV was 61.10 ± 0.33, 72.56 ± 0.45, 59.98 ± 0.55 and 62.65 ± 0.42 %, respectively; whereas at the same concentration activity of AA was 99.35 ± 0.38 % (Fig. 2a). The IC_{50} values of IRJ I, IRJ II, IRJ III, IRJ IV and AA were 52.38 ± 0.14, 37.98 ± 0.57, 55.90 ± 0.50, 50.93 ± 1.08 and 21.80 ± 0.30 µg/mL, respectively. IRJ II has shown higher scavenging activity compared to other IRJ samples (Fig. 2b).

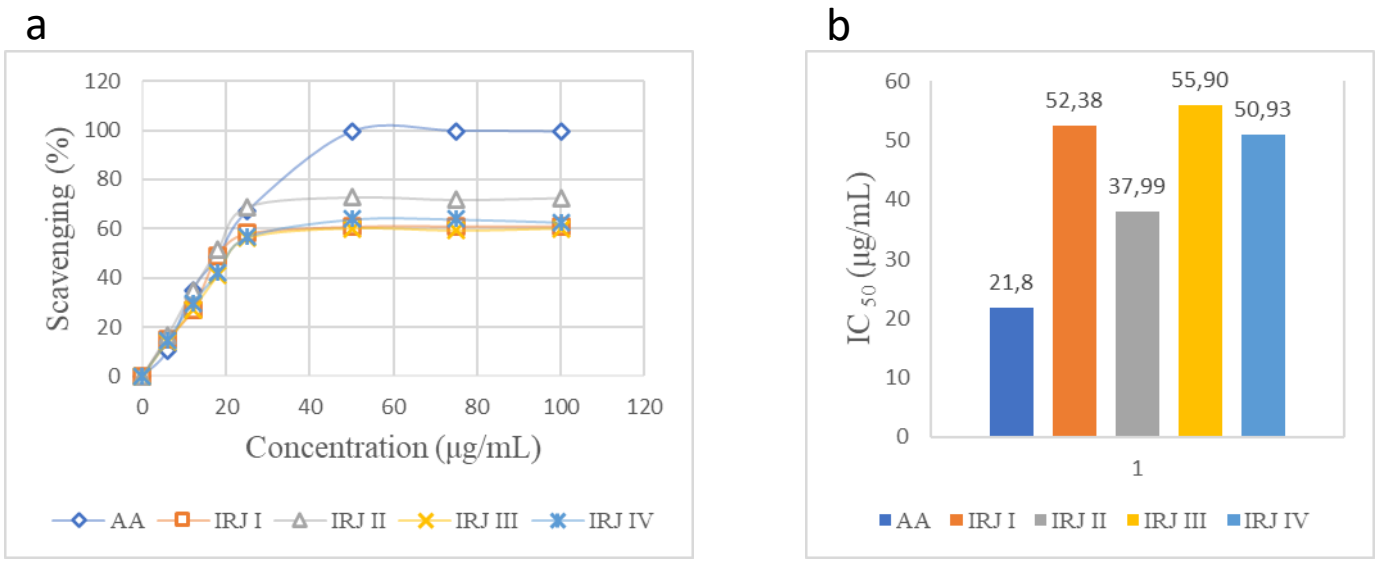

Figure 2. Determination of **a** ABTS inhibition activity and **b** IC_{50} of IRJ samples (I, II, III and IV). Data were expressed as a mean ± SD (n = 3, $p < 0.05$) for all samples.

2.5.3. *Reducing power (FRAP assay)*

The reducing ability of different IRJ samples were determined showing considerable antioxidant activity when compared with BHT. Reducing power of standard BHT was higher than IRJ samples. IRJ II has shown relatively higher activity amongst different IRJ Samples. The reducing potential of IRJ I, IRJ II, IRJ III and IRJ IV was found 58.20 ± 0.57,66.59 ± 0.08, 58.80 ± 0.16 and 61.34 ± 0.33 %, respectively at a concentration of 100 µg/mL, whereas, BHT's was 99.20 ± 0.83 % at the same concentration.

3. DISCUSSION

Interest in functional food has been steadily increasing over the years. Due to their nutritional and therapeutic benefits, these products are gaining intense commercial and research attention [17]. Numerous antioxidant formulations, especially those derived from natural sources have emerged on the market. One such value-added product is bee Royal Jelly, widely recognized for its therapeutic properties, including antioxidant activity. The present study reports the physiochemical properties, nutraceutical potential and antioxidant activity of various IRJ samples for the first time.

The published physicochemical parameters were compared with the present study and the results were analogous. As far as the composition of RJ is concerned, Kocot *et al*. [18], stated that the water content in the RJ is in a range of 50–70%. The total sugar content mainly consists of glucose and fructose, together these sugars account for 90% of all sugars and found in between 7-21.2% in RJ. Sucrose is always present along with other sugars but in variable concentrations 2.86% according to Kanelis *et al*. [19], 2.1% according to Kolayli *et al* [20], 0.5–2% according to Orsolic [21],, and 0.2% according to Wytrychowski [22]), these are closely associated with the sugar content of IRJ samples, despite the major sugar components being fairly less than the average values. Further, it was found that IRJ samples were rich in protein, had low carbohydrates and had high energy value indicating the rich nutraceutical potential of IRJ.

The investigations for TPC and TFC is important because of their strong correlation to antioxidant properties. However, based on the geographical source and bee species, the differences in the TPC and TFC of RJ samples have been reported. [23]. So, the IRJ samples from different geographical regions were examined, possessing considerable TPC and TFC. These findings indicate that IRJ could be a choice for commercial use because of the significant availability of polyphenols and flavonoids.

The free radical scavenging activity by DPPH method is one of the most used method for determining antioxidant activity. It is inexpensive, highly reproducible and can be developed rapidly. Thus, the antioxidant potential of IRJ samples were studied using the DPPH radical scavenging activity. Nagai et al. [16], [24], reported that water and alkaline extracts of RJ have free radical scavenging activity, which was attributed to protein and phenolic component of RJ. Present study showed significant DPPH radical scavenging activity by IRJ, which can again be associated to the high content of protein, phenolics as well as

flavanoids. Additionally TLC-DPPH scavenging activity of IRJ and 10-HDA was confirmed through yellow bands against purple background (Figure 3).

Figure 3. Track 1 represents 10-HDA and Track 2 represents IRJ II sample. Yellow spot against purple background conform antioxidant activity.

The major royal jelly proteins (MRJP) have been played to express antioxidant effect. Park MJ et. al. [25] observed that MRJP increased cell viability and reduced oxidative stress induced cell apoptosis. These protein showed direct shielding to the cell against oxidative stress and protect the mammalian and insect cells. Moreover MRJPs 1–7 exhibit DPPH free radical scavenging activity and protection against oxidative DNA damage. It is fairly believed that similar proteins present in our IRJ samples may be one of the key factors in exhibiting against oxidative stress. The lyophilized sample showed highest antioxidant effect, with the higher protein contents may be the imperative factor.

The reducing potential of a compound depends on the presence of reductones (antioxidants), which exert their antioxidant function by donating a hydrogen atom to break the free radical chain [26]. Reducing power of the samples increases with the increase in concentration. On comparing the results of IRJ samples, IRJ II exhibited the highest reducing power ability.

The 10-HDA, a quality parameter of RJ is also a key component in exhibiting the free radical scavenging effect [27]. The study correlated with IRJ samples in terms of scavenging potentials. In addition to it, the analytical study on the determination of HDA in various IRJ samples, previously determined by our research group, suitably correlated the antioxidant effect with the exact amount of HDA. Similarly the TLC-DPPH results of the present study confirm the radical scavenging activity of 10-HDA. Balkansa R et.al [15], reported that antioxidant activity of RJ is observed due to 10-HDA and polyphenol content. The relationship of total phenolic content and flavonoid content with IC_{50} value (DPPH and ABTS) of different IRJ samples is shown in Figures 4 and 5, respectively. The regression analysis showed the negative correlation between TPC and TFC with IC_{50}, which indicated the contribution of these component in antioxidant potential, evidently the remaining antioxidant activity come from other component. Thus present study concluded that the antioxidant activity of IRJ non-lyophilized samples as well as lyophilized sample is due to the composite effect of 10-HDA, TPC, TFC and other components present.

On keeping antioxidant potentials as the key parameter for the comparison, it was observed that IRJ samples excluding the lyophilized sample (IRJ) presented similar or overlapping free radical scavenging (DPPH or ABTS or Fe reducing) effect when compared with RJ extracted with absolute ethanol solely based on the ultrasound-assisted technology [28], Brazilian RJ [29,30], Turkish RJ [31], Italian RJ [32], Chinese RJ [33], Singaporean RJ [34], Japanese RJ [35], Bulgarian RJ [36], and Transylvanian RJ [37] in terms of approximately 90% limit. The overall findings of the study suggest that Indian Royal Jelly samples could be utilized as a promising nutritional product for mankind.

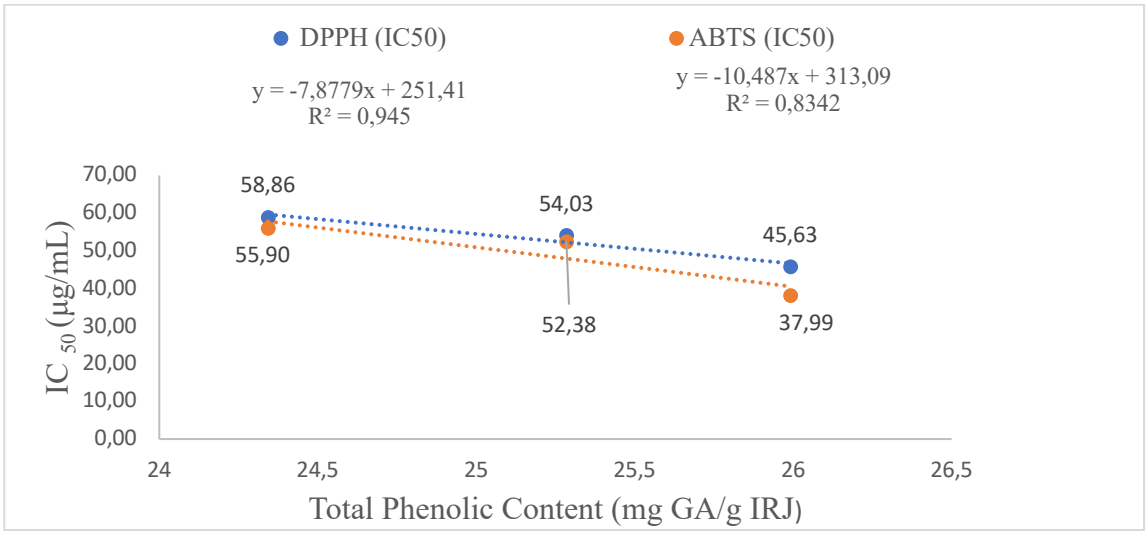

Figure 4. Relationship along with regression equation and correlation coefficient between IC_{50} and total phenolic content of IRJ sample. DPPH assay (Blue mark) indicated IC_{50}- 58.86 µg/mL of IRJ III, 54.03 µg/mL of IRJ I, 45.63 µg/mL of IRJ II. Similarly ABTS assay (Red mark) indicated IC_{50} -55.90 µg/mL, 52.38 µg/mL and 37.99 µg/mL of IRJ III, IRJ I, IRJ II respectively.

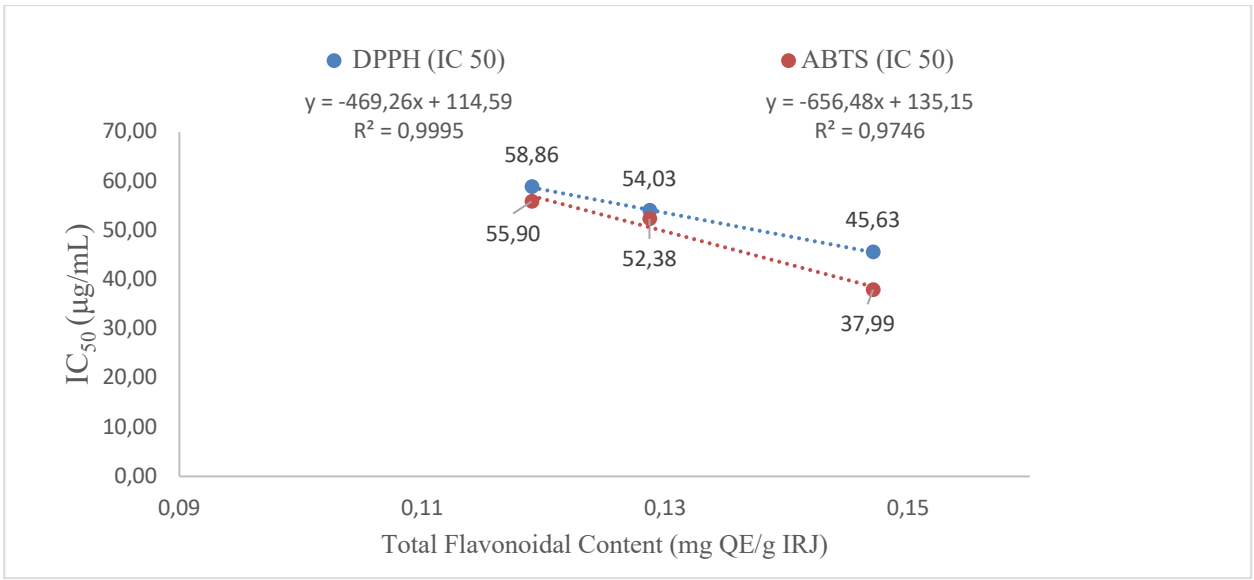

Figure 5. Relationship along with regression equation and correlation coefficient between IC_{50} and total Flavonoid of IRJ sample. DPPH assay (Blue mark) indicated IC_{50}- 58.86 µg/mL for IRJ III, 54.03 µg/mL for IRJ I, 45.63 µg/mL for IRJ II. Similarly ABTS assay (Red mark) indicated IC_{50} -55.90 µg/mL, 52.38 µg/mL and 37.99 µg/mL for IRJ III, IRJ I, IRJ II respectively.

4. CONCLUSION

In recent years, stressful work, unhealthy food and environmental pollution have increased free radical formation in the human body. The present study investigates IRJ as a potential nutraceutical agent. IRJ sample was composed of 11.2-12.97 % (w/w) of protein, 17.34-18.97 % (w/w) of total carbohydrates and 2.96-3.2 % (w/w) of fats. The IRJ can also be considered as an abundant source of calories. The study showed that IRJ is pesticide-free, making it safe to use. The present study also demonstrated the significant amount of polyphenolic content, suggesting IRJ is rich in antioxidants, its effectiveness for managing oxidative stress and have various beneficial impacts on human body. Therefore IRJ products can be included in the daily diet for a healthy life.

5. MATERIALS AND METHODS

5.1. Indian Royal Jelly samples

IRJ samples were collected from Southern, Northern and Central regions of India by Central Bee Research and Training Institute (CBRTI), Pune. Southern region sample was lyophilized. All the collected samples and lyophilized powder were supplied as a gift sample. The IRJ samples were weighed accurately and diluted with distilled water (10 mg/mL). After sonication for 60 minutes with occasional shaking, these solutions were centrifuged at 15000 rpm for 10 minutes, the supernatants were used for the following tests. The samples were designated as IRJ I to IRJ IV as shown in Table 1.

Table 1. List of IRJ samples along with sample code, sample type and code for Research studies

Sample Code	Sample type & region	Code for research studies
VIJ/F/1	Fresh IRJ, Southern region (SR)	IRJ-I
5/HA/2020	Fresh IRJ, Northern region (NR)	IRJ-II
4/MA/2019	Fresh IRJ, Central region (CR)	IRJ-III
VIJ/P/1	Lyophilized Powder-SR	IRJ-IV

5.2. Chemicals

DPPH (2, 2′-diphenyl-1-picrylhydrazyl), ABTS (2, 2′-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid), Trichloroacetic acid (TCA) and Folin-Ciocalteu's phenol reagent, bovine serum albumin were procured from Sigma-Aldrich Ltd., Bangalore. Gallic acid (GA), Ascorbic acid (AA), Potassium dihydrogen phosphate (KH_2PO_4), and dipotassium hydrogen phosphate (K_2HPO_4), sodium hydroxide, sodium bicarbonate, copper sulphate, sodium potassium tartrate, Aluminium chloride ($AlCl_3$) and solvents were procured from HiMedia Ltd., Mumbai.

5.3. Instrumentation

Spectrophotometric measurement was carried out using Jasco V-630 UV spectrophotometer. A Mettler Toledo® weighing balance, ATS-2-LCD Sonicator (Athena® Mumbai, India) were used during process. Eppendorf 5424R was employed at different stages of sample preparation. Nylon syringe filter (Chromatopak®, 0.22 µm) was utilized for filtration.

5.4. Physicochemical parameters

5.4.1. The nature, color, appearance, odor, and solubility (in water, ethanol, methanol, acetonitrile, and methanol: water) of the various IRJs were observed and reported.

5.4.2. Moisture content

Halogen moisture analyzer was used to analyze the moisture content of sample. IRJ Samples (~1 g) were placed in halogen moisture analyzer. In all cases, the samples were subjected to the process, until to get constant weight and the moisture content was calculated using equation:

$$\% \text{ Moisture} = (W_1 - W_2)*100$$

Where: W_1 = Total mass of the sample at the start of the process;
W_2 = Total mass of the sample at the end of process

5.4.3. Ash value

The accurately weighed sample of IRJ was placed into a muffle furnace at 600°C for 4 hrs in crucible, where it was charred. The total ash content of the IRJ was calculated by weighing the residue left behind in crucible using the formula:

$$\text{Total Ash value} = \frac{\text{Weight of residue after ashing}}{\text{Total weight of sample}} \times 100$$

5.5. Pesticide content

Agilent Technologies 6460 Triple Quad LC/MS was used to assess pesticide content. Water (8 mL) and acetonitrile containing 1% acetic acid (10 mL) were added to 2 g of sample. To this 1.5 g of anhydrous Sodium sulfate followed by 6 g of anhydrous magnesium sulfate were added. After 5 minutes of heating at 1500°C, the mixture was placed in a desiccator to cool. This was subjected to a 3-minute vortex and a 5-minute centrifugation at 4000 rpm, 5 mL of supernatant were transferred into a 15 mL polypropylene centrifuge tube, where they were agitated for 30 seconds, then centrifuged for five minutes at 10000 rpm. 2 mL of this supernatant were combined with 200 mL of 10% diethylene glycol solution and evaporated under nitrogen at 350°C until dryness was achieved. Reconstituted the sample with 1 mL of methanol and 1 mL of 0.1% acetic acid. This solution was filtered through a 0.2 µm membrane filter and injected (5-20 µL) into LC-MS/MS.

5.6. Chemical evaluation

The total polyphenol content and total flavonoid content were determined as per the method given by Nath *et al*., 2019 [13].

5.6.1. Total polyphenol content (TPC)

The Folin-Ciocalteu colorimetric technique was used to assess the total polyphenolic content of RJ's water-soluble extract, using gallic acid (GA) as a calibration reference. An aliquot of IRJ sample solution (0.1 mL) was mixed with water (5 mL) and Folin-Ciocalteu reagent (0.2 N) was then added (0.5 mL). After vortexing the mixture for 3 minutes, 1 mL aliquot was added to an aqueous Na_2CO_3 solution (35 g/L) and vortexed. The mixture was kept at room temperature for an hour. The absorbance was measured at 725 nm against a blank. Standard calibration curve (40 µg/mL to 1000 µg/mL) was prepared and used.

5.6.2. Total flavonoid content (TFC)

The method described by El-Guendouz et.al was used to determine total flavonoid content. 100 µL of IRJ samples were mixed with 100 µL of $AlCl_3$ (20%) and incubated for 1 hr. After incubation at room temperature, the absorbance was measured at 420 nm. Quercetin (QE) standard (10 µg/mL to 100 µg/mL) was prepared and used to calculate total flavonoid content per gram of IRJ (mg QE/g IRJ).

5.7. Nutraceutical parameters

The nutraceutical parameters of IRJ fat, protein, carbohydrate, and total energy were determined as per the method provided by Hu *et al*., 2021 [14].

5.7.1. Total fat content

IRJ 10 g and 1 g of sea sand were mixed together in a 500 ml beaker. Silica gel 60 was added to it and the mixture was homogenized. Silica gel 60 was filled in Soxhlet cartridge upto 1 cm height. The mixture was then added into the soxhlet cartridge and covered with cotton. Cartridge is placed onto soxhlet apparatus. Flask (M1) was weighed and used as a recovery flask. Petroleum ether: hexane (80:20) was used depending on volume of recovery flask, IRJ was extracted for three hours at 150^{0}C. Recovery flask was put into desiccator, cooled to room temperature and weighed (M2). Total fat was calculated by the formula:

$$\textbf{Total fat (g /100 g)} = \frac{M2-M1}{M1 \times 100}$$

5.7.2. Total protein content

The Lowry method was employed to determine the total protein content of IRJ by utilizing bovine serum albumin (BSA) as a reference. Weighed 10 mg of BSA and transferred into volumetric flask, 10 mL of double distilled water was added to dissolve it and made the final stock concentration of 1000 µg/mL with water. Further made concentrations ranging from 0.01 µg/mL to 100 µg/mL were prepared and aliquots were collected, Jasco V-630 UV-Vis spectrometer was used to evaluate the absorbance of BSA at 750 nm against a blank. 1 mL of IRJ was mixed and vortexed with 1.4 mL of Lowry solution (Mixture of Solution A:B:C in proportion of 100:1:1) (Solution A (500 mL)- 2.85 g of sodium hydroxide and 14.30 g of sodium bicarbonate, in distilled water, Solution B (100 mL) - 1.42 g copper sulphate in distilled water, and Solution C (100 mL) - 2.85 g of sodium potassium tartrate). The solution was kept at room temperature and in the dark for 20 minutes. Folin reagent was added to the sample and incubated for 30 minutes at room temperature in

the dark before being vortexed. The absorbance of the sample was measured at 750 nm against a blank solution. The protein content of the sample was determined using a standard calibration curve of BSA.

5.7.3. Total carbohydrate content

Total carbohydrate content of IRJ samples were determined using the following formula:

Carbohydrates (g) = 100 – [protein (g) + fat (g) + moisture (g) + ash (g)]

5.7.4. Total energy

The total energy of IRJ samples were calculated using the following formula based on the nutrient values of other components:

Energy (Kcal) = [4 × (protein (g) + carbohydrate (g) + 9 × fat (g)]

5.8. Antioxidant study

The IRJ samples were diluted with methanol and distilled water (50:50) (10 mg/mL). Sonicated for 60 min with occasional shaking. After sonication, the samples were centrifuged at 15000 rpm for 10 min, and the supernatants were used to determined antioxidant activity as per the method given by Balkanska *et al*., 2018 [15].

5.8.1. DPPH radical scavenging activity

The free radical scavenging activity of IRJ samples was based on the method of Nagai et al (16) with some modification. Different IRJ samples and 1.0 mM DPPH solution were prepared. To the various concentrations of IRJ solutions, 0.33 mL of DPPH radical solution and 2.40 mL of ethanol (99%) were added. The absorbance of solution was measured at 517 nm after vigorous shaking and keeping 30 minutes in the dark. Ascorbic acid (6 to 100 µg/mL) was used as a positive control. IC_{50} value of IRJ was estimated.
The DPPH scavenging capability of the IRJ was calculated as:

$$\textbf{DPPH Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

5.8.2. ABTS radical scavenging activity

The assay was performed as per El-Guendouz et al (3) ABTS radical was produced by reacting 7mM ABTS solution (aq) with $K_2S_2O_8$ (2.4 mM) solution in the dark for 16 hours, and adjusted the absorbance at 734 nm to 0.7 at room temperature, 350 µL IRJ solutions (6 to 100 µg/mL) were added with 650 µL of ABTS and the absorbance at 734 nm was measured after 6 minutes. The IC_{50} value of IRJ was estimated.
The scavenging capability the ABTS was calculated using the following formula:

$$\textbf{ABTS scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

5.8.3. Reducing power (FRAP assay)

The reducing ability of IRJ was determined by Oyaizu et.al. An equivalent amount of the 2.5 mL of IRJ sample solution was combined with 0.2 M sodium phosphate buffer (pH 6.6) and potassium ferricyanide (1%). The mixture was kept at 50°C for 20 minutes, which was then mixed with equal volume of trichloroacetic acid (1%) and centrifuged at 3500 rpm for 10 minutes. The supernatant was mixed with distilled water and ferric chloride (0.1%) in a 1:1:2 ratio. Measured the absorbance at 700 nm wavelength. Dibutyl hydroxytoluene (BHT) was utilized as a positive control. The measured absorbance was proportional to the reducing power of the IRJ samples.

5.9. TLC-DPPH assay

The TLC- DPPH assay technique was employed to confirm the antioxidant effect of Indian Royal Jelly sample. IRJ and 10-HDA were applied on the Silica gel 60 F254 TLC plates. Developed the TLC plates in a presaturated solvent chamber using toluene, methanol and ethyl acetate, (5:2:3) as a developing solvent. After the development, the plate was taken out of the chamber and sprayed with a 2.54 mM methanolic DPPH solution to derivatize them. On a purple backdrop, bands with DPPH scavenging activity may be seen as white and yellow stripes indicating the antioxidant potential of IRJ and 10 HDA.

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