https://doi.org/10.48047/AFJBS.6.13.2024.6420-6439

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Analysis of phytochemical and antimicrobial activities in Hassawi and Njavara rice varieties.

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Volume 6, Issue 13, Aug 2024

Received: 15 June 2024

Accepted: 25 July 2024

Published: 15 Aug 2024

doi: 10.48047/AFJBS.6.13.2024.6420-6439

Abstract

Sustainable agriculture entails native-adapted rice varieties that are highly nourished and intake limited agricultural inputs. Hassawi and Njavara (*Oryza sativa* L.) are reddish-brown rice varieties that contain several phytoconstituents, higher than hybrid rice varieties. The present study reveals that there is a higher content of carbohydrates, proteins, glycosides, tannins, saponins, alkaloids and flavonoids present in methanolic extract than in aqueous extract. Other secondary metabolites like, anthraquinones, phenolic compounds, fixed oils and fats, phytosterols and tannins were completely absent in both rice varieties of methanolic and aqueous extracts. Alkaloids are present more in Hasawi than in Njavara rice. Methanolic extracts show more inhibition activity compared to aqueous extracts respectively to the several bacterial species. Obtained results show phytoconstituents of both rice varieties are strongly inhibiting the *Bacillus subtilis* and *Bacillus cereus* and *Escherichia coli* and *Pseudomonas aeruginosa* growth. Njavara aqueous extract shows a maximum of 19.9 mm (500 ug/ ml) against *Bacillus subtilis.* But minimum zone of the inhibition 1.9 mm (1 ug/ml)] showed against *E.coli*. Our preliminary data reveals that Hassawi and Njavara rice varieties contain several phytoconstituents that could play a vital role in human health with huge supplementation of nourishment as a functional food.

Keywords: Brown rice, Methanolic extract, Phytochemicals, Flavonoids, Anti-microbial activity.

Introduction:

Native rice varieties are engrossed in significant consideration as of consumers owing to their unique biological activities and nourishment. Njavara also called Navara is a traditional rice variety in Kerala used in many ayurvedic medicines to treat Rheumatoid arthritis (as a paste called “Navarkazhi”) neurological, eye disorders, body pains, indigestion, respiratory disorders and increased lactation for feeding to new-born babies (Balachandran *et al.*, 2008).Hasawi Rice was cultivated in the Middle Eastern countries in the olden days, especially in Southern Iraq and spread to Iran with the advent of Islam ca 1400 ybp. Rice cultivation is predominated in Egypt's surroundings of Nile River delta regions, same like in Saudi Arabia cultivation of rice arose in the

AL-Ahsa region on the eastern side of the country and is therefore known as Hassawior Al-Hassawi rice (Al Mulla *et al.,* 2022)

Hassawi rice is adaptable to osmotic and drought conditions with undesired characteristics (susceptibility for lodging, delayed maturity and photoperiod sensitivity) in autumn (Al mssallem *et al*., 1997). This could be the main reason for growth in desert conditions. Earlier, studies show metabolites in 63 Asian pigment rice cultivars of Hassawi rich in antioxidants (proanthocyanidins), ash, non-starch polysaccharides, proximate fat, essential minerals (Z, Fe^{+2}, Ca^{+2}, Se, Sr, Mg^{+2} and Mn) and vitamins (B2, B6, C and E) than white rice (Sedeek *et al.,* 2023; Hadid and Elsheikh., 2012).These compounds possess anticancer, anti-inflammatory and antidiabetic properties and enhance the immune system to many chronic diseases (Rohrer & Siebenmorgen., 2004; Xu *et al.,* 2001). Hima and Deep (2018) noticed earlier that 113 volatile phytoconstituents contain medicinal activities in the Njavara rice variety, but most of them haven't been explored until now. Tannins from traditional rice varieties contain anti-diarrheal activities and may precipitate the enterocyte's periphery proteins to reduce peristaltic movement and intestinal secretion. In addition to that, saponins have hypoglycemic, cardiotonic and expectorant activities (Muthee *et al.,* 2016).

Secondary metabolites from traditional rice varieties can mitigate several abiotic and biotic stresses resulting from insects, pests and extreme environmental conditions (Aly *et al.*, 2013; Mohamed *et al.*, 2016; Jamloki *et al.,* 2021).Total flavonoid content (TFC) (153 to 771 mg /100 g) varied with bounded phenolics (102 to 443 mg/100 g), alkaloids and glycosides along with tannins. The high content of TFC is present in red and black rice varieties compared with white rice (Shen *et al*., 2009; Chandramouli *et al.*, 2017; Ali Ghasemzadeh *et al.,* 2018). Sometimes it assumes importance in comparing international rice variety (Hassawi) with wild variety (Njavara) of India to know about specific biochemicals and their associated activities. Such a kind of investigation may pave the way for improvement of Indian traditional varieties. In this preliminary study, we are reporting the analysis of Hassawi and Njavara rice's phytoconstituents and their activities.

Hassawi (*Oryza sativa L*.):

Kingdom : Plantae
Clade : Angiosperms
Clade : Monocots
Clade : Commelinids
Order :Poales
Family :Poaceae
Genus :*Oryza*
Species :*sativa*
Binomial name: *Oryza sativa Linn*.

Materials and methodology:

Seed collection and plant authentication:

The Hassawi grains were collected from a local farmer in Saudi Arabia and Njavara grains were obtained from Ulli Suresh, cultivator of indigenous rice varieties, in Uppada, Andhra Pradesh, India.

Extractions of plant material:

The aqueous and methanol extracts were prepared from fresh dehulled seeds and made into a fine-course powder. These powders were mixed with methanol and aqueous solutions with continuous shaking for 72 hours. Later, these extracts were filtered and crude extracts were generated with a rota evaporator. Extracts were stored at 4° C to avoid pathogen contamination until further experiments.

Preliminary phytochemical screening:

Hassawi and Njavara rice methanolic and aqueous crude extracts were used for primary-level phytochemical screening by high-standard protocols defined by Prabhavathi et al., 2016; Bhattacharya & Roy, 2018.

Methodology:

Total Alkaloids content:

The crude methanolic and aqueous extracts of two rice samples were separately treated with a few drops of diluted hydrochloric acid and filtered later. The filtrate was tested for the presence of alkaloids. Finally observed light brown colour indicates the presence of alkaloids in test samples.

Hager's test:

In this experiment, 2 ml of crude extracts were mixed with 5-7 drops of Hager's reagent (Picric acid solution) and observed yellow precipitation formation.

Mayer's test:

In Mayer's test, a few drops of Mayer's regent were added to 1 ml of plant extracts until yellowish or white precipitate observation noted.

Dragendrojf's test:

In this experiment, 2 ml of extracts were added into 1 ml of Dragendroffs reagent (Potassium bismuth iodide solution). Observed orange-red precipitation in test tubes and confirmed the presence of total alkaloids.

Wagner's test:

Extract treated with Wagner's reagent (Iodine-potassium solution) formed reddish brown precipitate.

Measurement of total Carbohydrates:

To estimate the total carbohydrate value in Hassawi and Njavara rice varieties, crude extracts were dissolved in 5 ml of doubled distilled water and filtered, the obtained solution was used for carbohydrate quantification.

Molisch's test:

2 ml of extracts were treated with 6-8 ml of Molisch reagent (alpha naphthol in 95% ethanol) and later few drops of concentrated Hydrochloric acid were added along the wall of the test tube. Immediately, observed a violet-coloured ring formation at the junction of two reagents indicating that carbohydrates were present in the samples.

Benedict's test-

Both, Hassawi and Najavara extracts were treated with 5-10 ml of Benedict reagent (Copper Sulfate, Sodium Citrate and Sodium Carbonate in water) was added into 8-10 ml of rice extract and heated for five minutes and observed dark red precipitation indicating the presence of carbohydrates.

Fehling's test:

2 ml of extracts were treated with an equal amount of Fehling's reagent A (Copper Sulfate in water), and Fehling's reagent B (Sodium Potassium Tartrate) and heated for five minutes and observed to dark red colour.

Barfoed's test-

Extract treated with Bradford reagent (Copper Acetate in water and Glacial Acetate) red colour formation noted.

Measurement of total Glycosides:

Rice extracts were separately hydrolysed with diluted Hydrochloric acid for ten minutes in a water bath and then subjected to carbon tetrachloride, later added equal concentration of ammonium reagent and shaken well. The formation of a blue colour is an indication of the presence of cardiac glycosides.

Libermann Burchard's test:

Extract treated with Chloroform in a dry test tube and a few drops of Glacial Acetic Acid and a few drops of concentrated Sulphuric Acid at the sides

of the test tube. A red colour at the junction of two layers and the upper layer shows a green colour.

Legal test-

Extract treated with Disodium Nitroprusside in pyridine and Sodium hydroxide, red colour formation takes place.

Bomtrager's test-

Extract treated with diluted Sulphuric Acid add Ether are shaken and filtered. Addition of Ammonium solution to the obtained organic layer turns the layer to pink.

Measurement of total Phytosterols:

Reflux the extracts with alcoholic Potassium Hydroxide till complete saponification takes place. Dilute the saponification mixture with distilled water and extract with ether.

Evaporate the ethereal extract and subject the residue to the following tests

Liberman Burchard's test:

Extract treated with chloroform in a dry test tube and a few drops of glacial acetic acid and a few drops of concentrated Sulphuric Acid at the side of the test tube. A red colour at the junction of two layers and the upper layer shows a green colour.

Salkowski test:

Extract treated with equal volumes of Chloroform and Sulphuric Acid and appeared as a red or violet colour.

Measurement of total saponins:

Foam test:

Dilute l ml of each extract separately with double distilled water unto20ml (final volume) and applied continuously vigorous shaking for up to more than 5 minutes and kept rest for five minutes. After five minutes a one-centimetre thickness of foam formation indicated the saponins presence in rice samples.

Haemolysis test:

In this test, 2ml of 1.8% Sodium Chloride solution was taken in two test tubes. To one test tube, 2ml of distilled water was added and to the other 2 ml of 1% filtrate. Blood is obtained by pricking the thumb and 5 drops of blood were added to each tube, the contents were gently mixed and observed under a microscope. Haemolysis occurs, indicating the presence of saponins.

Measurement of total Tannins:

Dilute small quantities of extracts separately with distilled water and subjected to

Ferric chloride test:

In this experiment, 1 ml of Methanol and aqueous extracts were treated with 2 ml of 5 % Ferric Chloride solution and observed dark blue colour in test samples indicating the presence of tannins.

Gelatin test:

Extract treated with gelatin solution show white precipitate.

Lead acetate test-

One millilitre of lead tetra acetate was treated with 0.5 ml of rice extracts, and after some time yellow precipitate formation was identified as a tannin existence.

Measurement of total proteins and amino acids:

Dissolve small quantities of extracts separately with a few ml of distilled water and then subject to

Millions test:

Extract treated with Million's reagent (Mercuric nitrate in nitric acid), red colour appears.

Biuret test:

1 ml of rice extracts was mixed with 2 ml of Copper Sulphate and 5-6 ml of 10 % NaoH. Red or violet colour observation, indicating the presence of proteins in rice samples.

Ninhydrin test:

Freshly prepared 2 ml of Ninhydrin (0.2%) reagent is added to 1 ml of rice extracts and observed purple colour indicating the presence of overall proteins.

Sodium Bicarbonate test:

Extract treated with Sodium Bicarbonate solution, brisk effervesces.

Measurement of total Flavonoids:

Ferric chloride test:

To alcoholic extract, few drops of neutral ferric chloride solution addition forms blackish red colour.

Lead acetate test:

To the alcoholic extract, added Lead Acetate solution to form yellow precipitate,

Shinod's test:

In shinod's test, 8-10 drops of 100 % HCl were slowly mixed with a few fragments of Magnesium ribbon along with 1 ml of extract and after a few minutes observed pink colour indicates the presence of total flavonoids.

Zinc-hydrochloric acid test:

To alcoholic extract, a pinch of zinc dust was added and concentrated Hydrochloric acid was added along the side of the test tubes, Magenta colour formation noted.

Alkaline reagent test:

Crude extract residues were treated with 2-3 drops of diluted NaOH along with diluted HCl (80%), and solubility and colouration were observed. Initially, dark yellow-coloured precipitation appeared and it turned colourless with the addition of more diluted HCL confirming the presence of the flavonoid.

Anthraquinones (Borntgers Test):

0.5 g of rice methanolic and aqueous extracts were taken into a dry test tube containing 3 ml of Chloroform mixed well for 5 min and filtered with filter paper. This filtered solution was added with an equal amount of 10% Ammonia solution and observed violet colour formation in the lower side of the Ammonia layer confirming the anthraquinones.

Measurement of total Terpenoids:

A volume of 5 ml of the plant extract was mixed in 2 ml of chloroform and concentrated H_2S0_4 was added to form a layer. A reddish-brown colouration of the interface was formed indicating the presence of terpenoids.

Cardiac Glycoside (Keller-Killani) Test:

The extract was dissolved in 2ml of Chloroform and Sulphuric acid, formation of a brown ring at interphase appeared, confirming the presence of cardiac glycoside.

Fixed oils and fats:

A drop of concentrated extracts was pressed in between two filter papers and kept undisturbed. Oil strains on the paper indicate the presence of oils and fats.

Measurement of total Phenolic compounds:

Ferric chloride test:

When the extract treated with ferric chloride solution, the blue colour appears if hydrolysable tannins are present and a green colour appears if condensed tannins are present.

Gelatin test:

In ths test, extracts were added into a solution containing1% Gelatin along with 10% NaCl.

Antibacterial screening:

Media preparation:

The agar well diffusion method was followed for screening of antimicrobial activities with Hassawi and Najavara methanolic and aqueous extracts. Logarithmic phased pathogen species like *Bacillus subtilis* and *Bacillus cereus* (gram-positive) and *Escherichia coli* and *Pseudomonas aeruginosa* (gram-negative) inoculums were inoculated into NAM (Nutrient agar media). Upon proper solidification, wells were made using a sterile cork borer (6 mm in diameter) into agar plates. Then, 150 ul of methanolic and aqueous extracts were added to 1 ug/mL, 50 ug/mL, 100 ug/mL, 250 ug/mL and 500 ug/mL concentrations and gentamycin was employed as a positive control in respective wells. This concentration of extracts has been finalised based on present experimental conditions and previous literature. Plates were incubated at 37°C for 24 hours for proper bacterial growth. Antimicrobial activity was detected by measuring the zone of inhibition (including the walls of diameter) that appeared after incubation.

Results

S. No.	Phytoconstituent	Tests	Njavara		Hassawi	
			Methanol	Water	Methanol	Water
1	Alkaloids	Hagner's test	+	--	++	--
		Mayer's test	+	--	++	--
		Dragendrojf's test	+	--	++	--
		Wagner's test	+	--	++	--
2	Amino acids	Millions test	++	--	++	--
		Ninhydrin test	++	--	++	--
3	Anthraquinones	Borntgers Test	--	--	--	--
4	Carbohydrates	Molisch's test	+	--	++	+
		Fehling's test	+	--	++	++
		Barfoed's test	++	--	++	--
		Benedict's test	++	--	++	--
5	Cardiac Glycoside	Legal test	++	--	++	--
		Bomtrager's test	++	--	++	--
6	Fixed oils and fats	Ferric chloride test	+	+	--	+
7	Flavonoids	Lead acetate test	++	--	++	--
		Shinda's test	++	--	++	--
		NaoH test	++	--	++	--
		Zinc hydrochloride test	++	+	++	+
		Ferric chloride test	--	--	--	--
8	Phenolic Compounds	Gelatin test	++	+	++	+
		Liberman Burchard's test	--	--	--	--
9	Phytosterols	Salkowski test	++	--	++	++
		Biuret test	+	--	+	--
10	Proteins	Sodium bicarbonate test	++	+	++	+
		Haemolysis test	+	--	+	--
11	Saponins	Foam test				
		Cardiac Glycoside (Keller-Killani) Test	++	+	++	+
12	Terpenoids	Ferric chloride test	--	--	--	--
13	Tannins	Gelatin test	++	+	++	+
		Lead acetate test	++	+	++	+

Table 1: Identification of phytoconstituents in methanolic and aqueous extracts of Hasswai and Njavara rice varieties.

Preliminary phytochemical screening:

In this preliminary investigation, we found various types of phytoconstituents present in Hassawi and Njavara crude extracts. As per our observation, there is a higher content of carbohydrates, proteins, glycosides, tannins, saponins, alkaloids and flavonoids present in methanolic extract than in aqueous extract. Other secondary metabolites like, anthraquinones, phenolic compounds, fixed oils and fats, phytosterols and tannins were completely absent in both rice varieties of methanolic and aqueous extracts. Alkaloids are present more in Hasawi than in Njavara rice. This data reveals the extraction of phytoconstituents present in higher concentrations in methanolic extract than in aqueous extracts. These could also depend upon the polarity of solvents and rice cultivar and water solubility (Table 1).

Antimicrobial activity:

We observed both the Hasswai and Njavara rice varieties contain antimicrobial properties. Methanolic extracts show more inhibition activity compared to aqueous extracts respectively to the several bacterial species. Obtained results show phytoconstituents strongly inhibiting the *Bacillus subtilis* and *Bacillus cereus* (gram-positive) and *Escherichia coli* and *Pseudomonas aeruginosa* (gram-negative) growth. Njavara aqueous extract shows a maximum of 19.9 mm (500 ug/ ml) against *Bacillus subtilis.* But the minimum zone of the inhibition 1.9 mm (1 ug/ml)] was noticed against *E.coli*. Similarly, different zones of inhibitions were observed based on their relative concentrations in *Bacillus subtilis* and *Pseudomonas aeruginosa* (Table 2; Fig 1-4).

In *B.subtilis*, the zone of inhibition is completely absent at 1 ug/ ml concentration in all extracts.

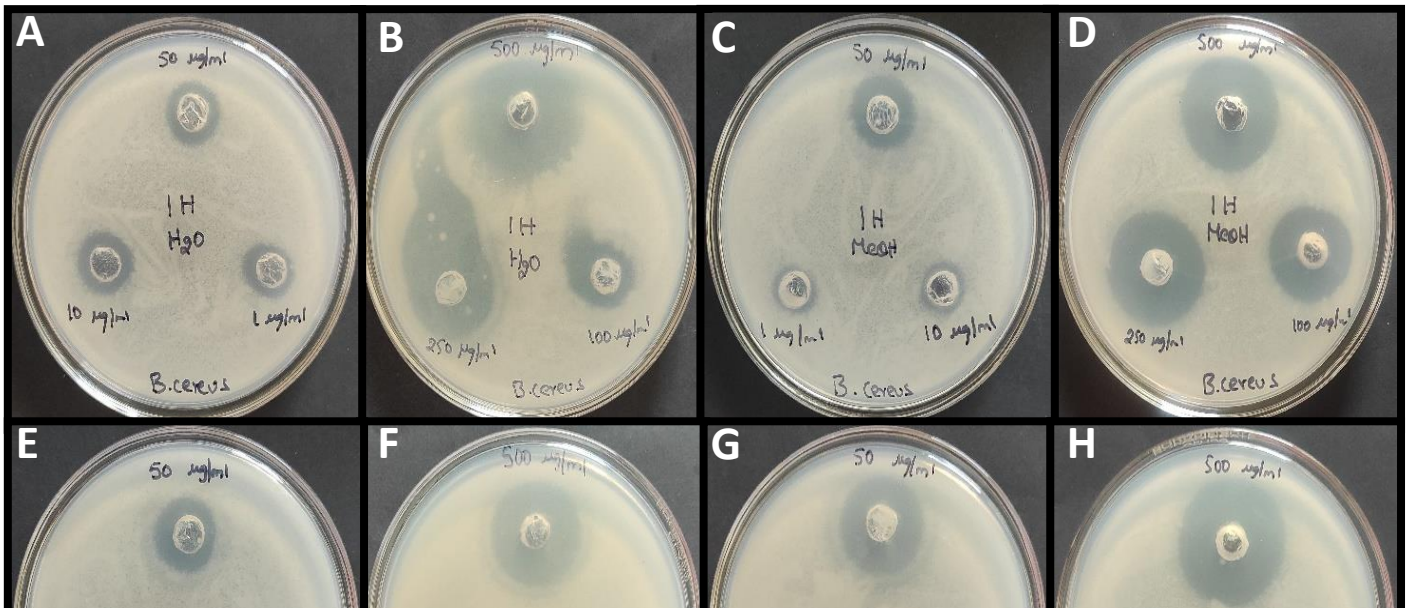


Fig1: Anti-bacterial activity of Hassawi and Njavara methanolic and aqueous extracts against *B.cereus*.

Fig2: Anti-bacterial activity of Hassawi and Njavara methanolic and aqueous

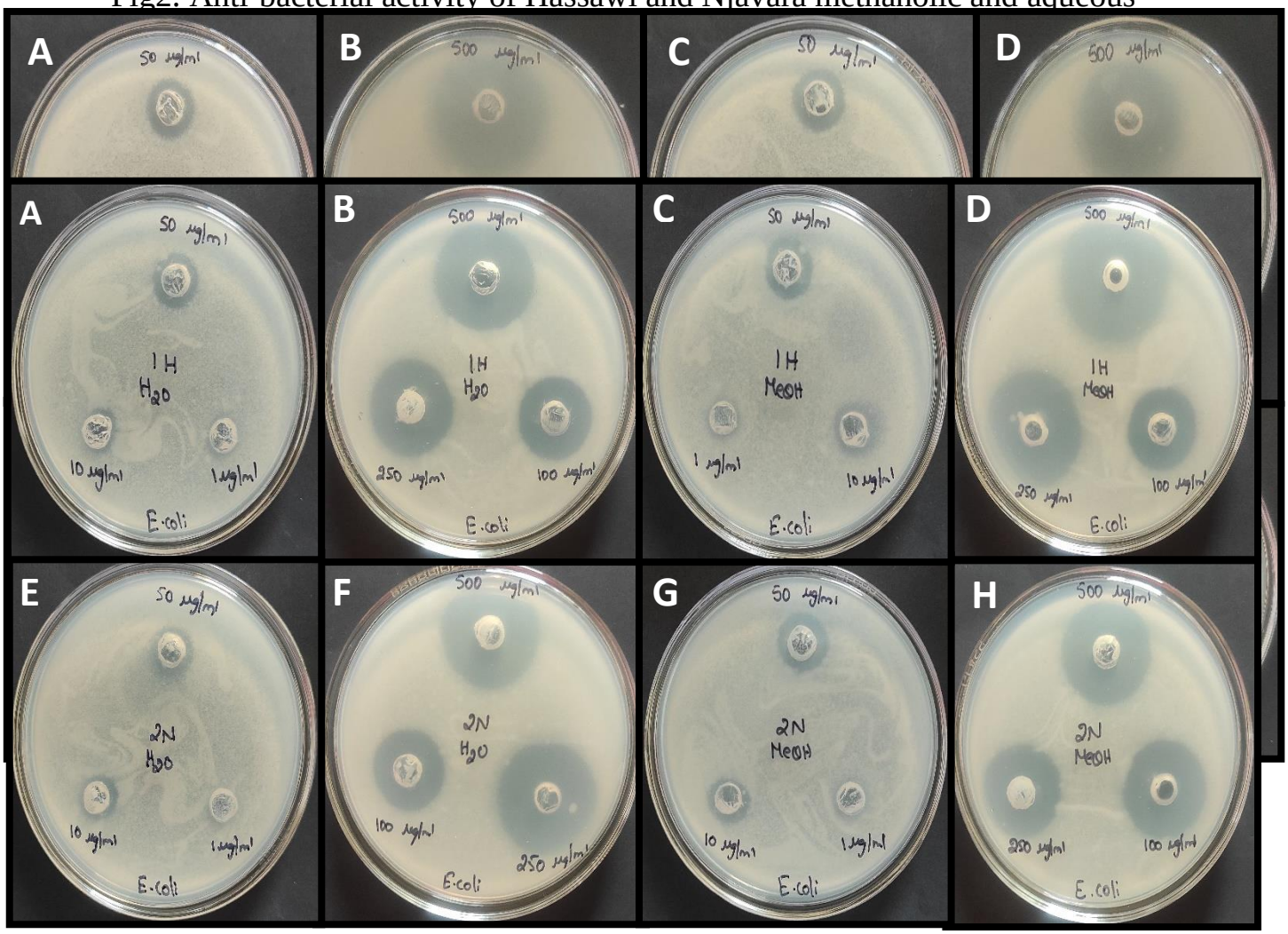


Fig3: Anti-bacterial activity of Hasawi and Njavara methanolic and aqueous extracts against *E. coli.*

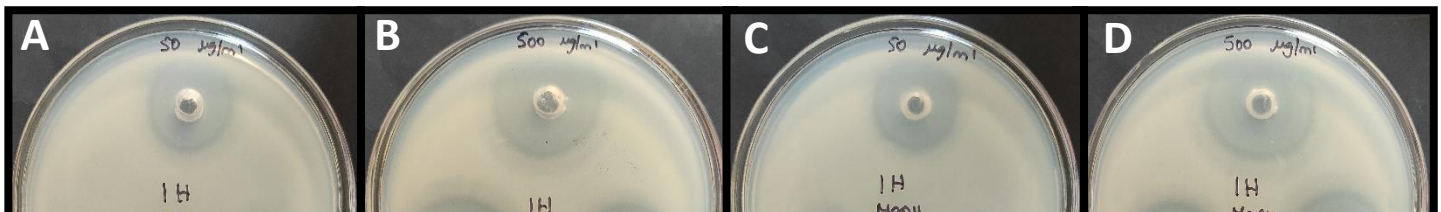
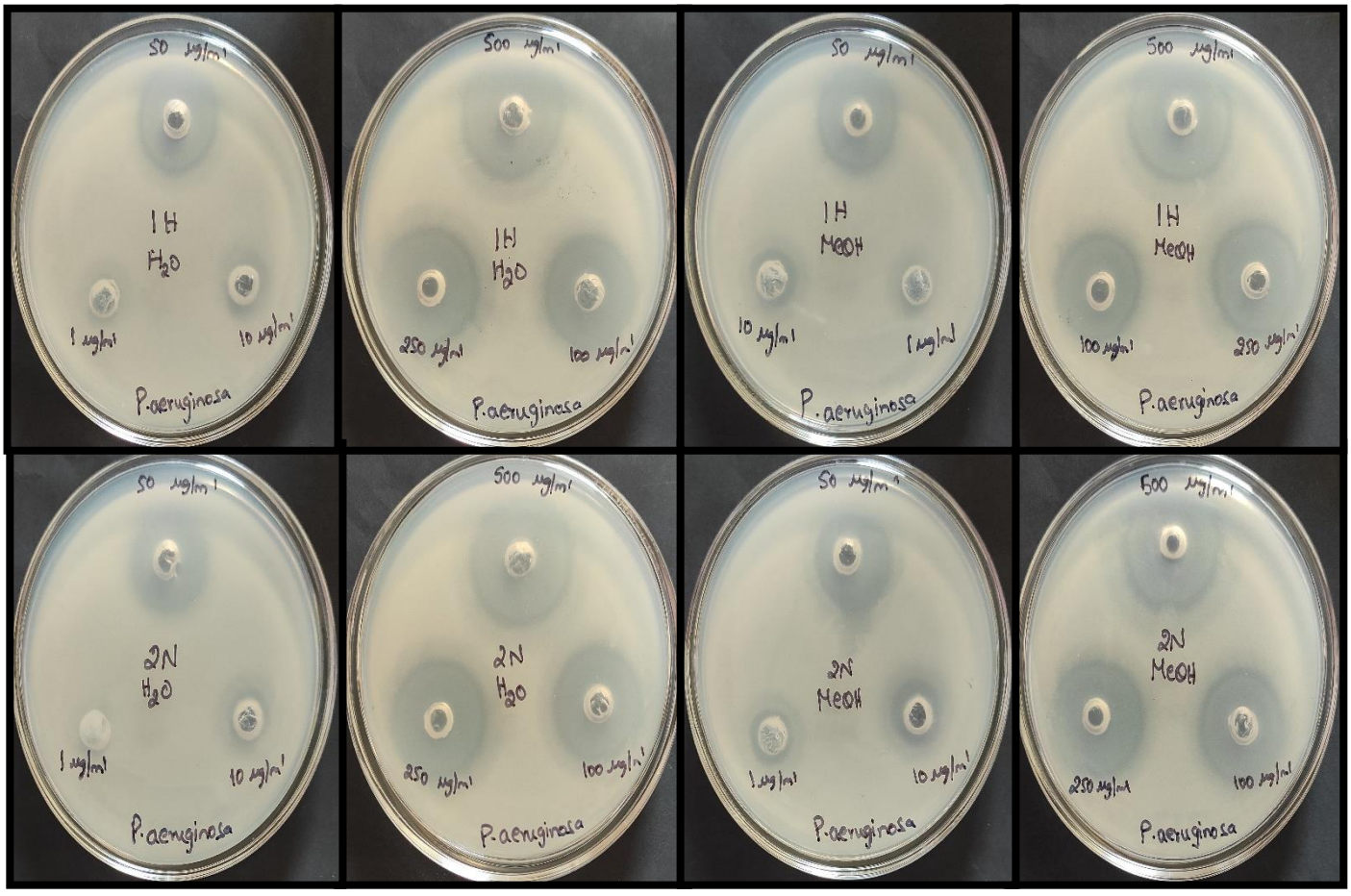


Fig4: Anti-bacterial activity of Hassawi and Njavara methanolic and aqueous extracts against *P.aeruginosa*



S. No	Name	Sample	Zone of inhibition (mm)					
			500 µg/mL	250 µg/mL	100 µg/mL	50 µg/mL	10 µg/mL	1 µg/mL
1	*Bacillus cereus*	1H Methanolic extract	15.5	15.0	14.9	7.2	3.1	2.0
		1HWater extract	19.5	15.8	11.2	6.9	3.5	2.3
		2N Methanolic extract	19.8	16.5	15.1	9.5	3.6	2.2
		2N Water extract	15.9	14.7	11.1	6.8	3.7	2.0
2	*Bacillus subtilis*	1H Methanolic extract	18.5	16.8	14.9	5.3	2.1	--
		1H Water extract	19.8	17.6	15.2	2.2	--	--
		2N Methanolic extract	19.2	16.7	15.6	5.5	2.9	--
		2N Water extract	19.9	16.4	11.3	2.2	--	--
3	*Escherichia coli*	1H Methanolic extract	18.4	15.9	11.7	4.3	3.2	2.1
		1HWater extract	16.9	15.8	12.7	3.6	2.2	--
		2N Methanolic extract	17.1	14.8	14.5	5.4	3.3	1.9
		2NWater extract	17.8	16.9	10.5	5.5	2.2	--
4	*Pseudomonas aeruginosa*	1H Methanolic extract	17.3	15.2	13.6	10.5	3.6	2.1
		1H Water extract	17.9	15.3	13.8	10.9	2.8	--
		2N Methanolic extract	18.2	17.9	13.1	8.5	5.2	2.2
		2N Water extract	18.1	17.3	13.4	9.2	2.9	--

Table 2: Antibacterial activity of Hassawi and Njavara rice varieties against bacterial species. 1. *Pseudomonas aeruginosa*, 2.*Escherichia coli*, 3. *Bacillus subtilis*, 4. *Bacillus cereus.*

Discussion: The present preliminary investigation is carried with an aim of finding the phytoconstituents and associated activities in one international (Hassawi) and Indian traditional varieties (Njavara). Indeed we have obtained significant results (Table 1 and 2) and are discussed in light of available literature below. Several traditional pigmented rice varieties contain nearly 2800 mg /100 g protein content. Proteins in pigmented rice varieties also reduce the allergy. Like that, there is a maximum protein content of 2900mg/100g in Njavara rice (Safia*et al.,* 2021; Kaur *et al.,* 2018).Likewise; we determined overall proteins in Hassawi and Njavara rice varieties which were involved in several biological activities for enhancing the nutrient supplementation and health benefits. Our results were consistent with previous findings, in those phenolics and flavonoids in cereals were primarily dispersed in the free form (Zhang *et al.*, 2010; Ti *et al.*, 2014). In traditional rice varieties, these glycosides were linked to various carbohydrate moieties, organic acids, phenols and lipids (Butsat *et al.*, 2010)

The phytochemical analysis of the hexane, methanol, ethyl acetate, acetone and water extractsshow that flavonoids, saponins, glycosides, tannins, terpenoids, steroids and phenolic compounds are present in satisfactory sums in the grains, leaves and stem of the Hassawi and Njavara rice varieties. Safia *et al.*, (2021) noticed that, there is an 85 % (13.36 mg/100 g) higher anthocyanin content in pigmented rice compared with white rice (2.04 3g/ 100g) but, Njavara shows the highest TAC values (19 mg/100g). In this work, we identified that Hassawi and Njavara both contain anthocyanins. Our data shows similarity with previous studies conducted by Oki *et al* (2002) and reported a strong correlation between pro anthocyanidins content in red pericarp and antioxidant activity. Proanthocyanidins in pigmented rice varieties protect against type-II diabetes, inflammation and cancer(Chen *et al.*, 2016).Similarly, anthocyanins in black rice possess hypoglycemic activity (Tsuda *et al.*, 2003).Our results also showing the presence of several anthocyanins in both traditional rice varieties that may control diabetic and cancer activities.

The average flavonoid content in pigmented (black and red) rice varieties (1308mg/100g) compared with non-pigmented rice is 46% (701 mg/100g) lower. In Njavara, high flavonoid content (1750 mg/100 g) reported (Safia *et al.*, 2021). Earlier, Shylaraj *et al*., (2017) noticed that, several kinds of saturated fatty acids (19% - 24%), monosaturated fatty acids (44% - 48%) and polyunsaturated fatty acids (30% - 33%) are present in traditional rice varieties. In addition to these compounds, ethyl ester (lauric acid ester), octadecanoic acid and ethyl esters increase the low-density lipoprotein (LDL) in the human body (Sulochana *et al*., 2016). Non-starch polysaccharides (NSPs) have 548% higher

content along with Selenium in Hassawi than Basmati and Brown rice (Muneera*et al.,* 2020).This will explain unprocessed rice with an outer bran layer before consumption.

Paul and Moolan (2014) reported earlier that Njavara methanolic and ethanolic extracts contain antimicrobial properties. Our data also matched with Chandramouli *et al.,* (2017) reported earlier, that methanolic and aqueous extracts show strong inhibition against *Staphylococcus pyogenes*, *Salmonella* and *E. coli.*This anti-pathogenicity data suggests that saponins, tannins, alkaloids, flavonoids and steroids from Hassawi and Njavara rice varieties are actively involved in the inhibition activity of several pathogens. Using these rice varieties we can also develop natural food preservatives against bacteria.

Conclusion:

The present preliminary phytochemical investigation showed the presence of various phytoconstituents in Hassawi and Njavara rice varieties that could have played an important role in pharmacological activities. This study confirms that Hassawi and Najavara varieties are rich sources of carbohydrates, proteins, glycosides, tannins, saponins, alkaloids and flavonoids. These metabolites play an important role in antibacterial activity against *Bacillus subtilis* and *Bacillus cereus*, *E.coli* and *Pseudomonas aeruginosa*. The prominent nutritional richness can be achieved with pigmented rice to mitigate malnutrition. The cultivation of these Hassawi and Njavara rice varieties has the potential to enhance food security.

Declaration of competing interest:

All authors read and approved the final manuscript and declare that there is no conflict of interest.

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