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Phytochemical Analysis and Biological Activity of Malaysian *In Vitro* Cultured Aromatic Rice (*Oryza sativa* L. Cv. MRQ 74)

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This study aimed at screening the phytochemical content of *in vitro* grown *Oryza sativa* L. Cv. MRQ 74 and to determine its anti-cancer and cytotoxicity properties. The dehusked seeds of *Oryza sativa* L. Cv. MRQ 74 were surface sterilised using Clorox and Tween 20. The surface sterilised seeds were then cultured into MS media. Stems of 6-week-old aseptic seedlings were cultured on MS media with and without BAP hormones. The explants were cultured onto MS media fortified with 0.1 mg/L BAP and hormone-free as a control. Crude extracts of the stems and leaves of raised plantlets were prepared by maceration. Phytochemical screenings were conducted to detect the presence of flavonoids, alkaloids, saponins, tannins, terpenoids, resins, phenols, reducing sugar and iodine. Anti-cancer and cytotoxicity tests were done on HepG2 and Vero cells using MTT assays. The results revealed that the crude extracts derived from plantlets grown on MS media with and without BAP were found to be rich in saponins, terpenoids, resins and reducing sugar. There was no evidence of flavonoids, alkaloids, tannins, carbohydrates or starch in both crude extracts. BAP crude extracts have potential as an anti-cancer agent as they only require low concentrations to inhibit the growth of HepG2 cells.

Key words: Aromatic rice; *In vitro* cultured; BAP hormone; Phytochemical screening

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Plant phytochemicals are vital as they can be used in human therapy, veterinary, agriculture, scientific research and countless other areas [1, 2]. Phytochemicals have been used by humans since prehistoric times. Plant products can be derived from barks, leaves, flowers, roots, fruits, and seeds [3]. Phytochemicals have been detected in many plant species, for instance, *Ipomea aquatica*, *Tinospora cordifolia* and *Xanthium strumarium* [4]. Abeer et al. reported that terpenes, cardiac glycosides and carbohydrates were present in the aqueous extract of *Faidherbia albida* [5]. Plants are the primary source of bioactive compounds that have medicinal properties. Plant-derived products are most likely to be chosen by the consumer as they are safer, eco-friendly, and cost-effective. Several medicinal plant species have been shown to inhibit the progression and development of cancer. Currently, chemotherapy is one of the methods used for cancer treatment. Nevertheless, this method may damage healthy cells because chemotherapy drugs are unable to selectively target cancer cells. Among the side effects of cancer treatment are nausea, vomiting, hair loss, fatigue and memory problems. Hence, an efficacious natural product that has minimal side effects is desired. Therefore, finding new anti-cancer compounds from medicinal herbs are vital [6]. Yu et al. reported that rice bran extracts and fermented rice bran played an important role in hindering cancer development through downregulation of the inflammatory response [7]. The focus of our project was to evaluate the anti-cancer and cytotoxicity properties of the crude extract of *Oryza sativa* L. Cv. MRQ 74.

Rice (*Oryza sativa* L.) is one of the most important food crops globally. There are many varieties of cultivated rice in Malaysia such as MR 216 and MR 278. However, Malaysian aromatic rice cultivar MRQ 74, locally known as 'Mas Wangi', is the most preferred due to its good quality and low glycaemic index which is beneficial to diabetic patients [8]. Moreover, MRQ 74 cultivar is resistant to drought, pests and diseases. However, research on phytochemicals, cytotoxicity and anti-cancer agents related to staple food crops such as *in vitro* grown rice is still scarce. In the present study, the phytochemical analysis of *in vitro* raised plantlets on Murashige and Skoog (MS) media with and without the presence of

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plant growth hormones was conducted and compared. Anti-cancer and cytotoxicity tests were also performed on the crude plant extracts.

MATERIALS AND METHODS

Collection of Plant Materials

Mature rice seeds (*Oryza sativa* L. Cv. MRQ 74) were obtained from the Malaysian Agricultural Research and Development Institute (MARDI) Seberang Prai, Penang, Malaysia.

Surface Sterilisation of Rice Seeds

Mature rice seeds in healthy condition were selected by physical appearance and manually dehusked. The dehusked seeds were surface sterilised by soaking and shaking in 70% (v/v) Clorox with two drops of 1 mL/L Tween 20 followed by 50%, 30%, 20% and 10% (v/v) Clorox. The seeds were then rinsed once in sterilised distilled water. Finally, the seeds were rinsed in 70% (v/v) ethanol for one minute, followed by sterilised distilled water three times for complete removal of Clorox and ethanol, in a laminar air flow cabinet. The sterilised seeds were then cultured onto an MS medium containing sucrose (30 g/L) and agar (8 g/L).

Preparation of Culture Media

The MS medium was prepared by mixing 30 g sucrose in 1 L distilled water with the addition of 4.4 g of MS powder [9]. The pH of the MS medium was adjusted to 5.8 using 0.5 M sodium hydroxide (NaOH) and 0.5 M hydrochloric acid (HCl) before autoclaving at 121 °C for 20 minutes. The medium was poured into sterile gem jars and left to solidify at room temperature. The sterilised seeds were then cultured onto the MS medium in a laminar airflow cabinet. The prepared cultures were incubated in the culture room at 25 ± 1 °C, under light for 16 hours and subsequently 8 hours in the dark, daily for 6 weeks. The seeds started to germinate after 3 days. The seedlings produced were used as explant sources.

In vitro Propagation

The stem explants of 6-week-old aseptic rice seedlings were cut into approximately 5.0 to 10.0 mm segments and were cultured onto an MS medium containing 30 g sucrose and 8 g technical agar fortified with 0.1 mg/L BAP. Simultaneously, the stem explants were also cultured on a hormone-free MS medium as a control. The cultures were maintained in the culture room at 25 ± 1 °C, under light for 16 hours and subsequently 8 hours in the dark, daily for 6 weeks.

Extraction of Plant Materials

Six-week-old *in-vitro* plant leaves and stems of *Oryza sativa L.* Cv. MRQ 74 were dried in an oven at 40 °C for one night and then cut into chunks and ground using a dry blender. For the maceration process, approximately 20 g of the ground materials were mixed with just enough chloroform to cover the material and left for two days at room temperature to extract moderately non-polar compounds. The filtration process was carried out to separate the rice plant extracts (supernatant) and then the supernatant was removed using a rotary evaporator to eliminate unwanted solvent. Thus, the rice plant extracts were collected and stored in a small vial. The used ground material in the previous step was utilised again in the maceration process with methanol as the solvent to extract polar compounds. The same procedures were followed. Each step was repeated three times to obtain the maximum amount of extract.

Phytochemical Screening of Plants

The preliminary qualitative screening of chemical constituents was performed using the following test methods [2,10]:

Shinoda Test

The crude extract was mixed with a few fragments of a magnesium metal ribbon and concentrated HCl was added dropwise into the mixture. The appearance of a pink or red colour after a few minutes indicated the presence of flavonoids.

Mayer's Test

The crude extract was mixed with 2 mL of 1% HCl and heated gently. Mayer's reagent was then added to the mixture. A turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Saponins Test

The crude extract was mixed with 5 mL of distilled water in a test tube and shaken vigorously. The formation of a stable foam was taken as an indication of the presence of saponins.

Tannins Test

Two millilitres of the crude extract was mixed with ethanol in a test tube. About 3.5 mL of distilled water was added and the mixture was shaken. The presence of tannins was indicated by the formation of a green or blue colour.

Terpenoids Test

The crude extract was dissolved in 2 mL of chloroform and evaporated to dryness. Two millilitres of concentrated sulphuric acid (H_2SO_4) were added and the mixture was heated for 2 minutes. A reddish-brown colour indicated the presence of terpenoids.

Resins Test

Five millilitres of copper acetate were mixed with 5 mL of the crude extract. The mixture was shaken vigorously. The formation of a green solution confirmed the presence of resin.

Tannin and Phenol Tests

Ferric chloride solution was mixed with 2 mL of the crude extract. A green or dirty green precipitate indicated the presence of phenolic and tannin compounds.

Fehling's Test

Two millilitres of Fehling 1 and 2 reagents were mixed in equal volume. Approximately 2 mL of the crude extract were added into the solution in the test tube. The mixture was boiled gently. The appearance of a brick-red precipitate at the bottom of the test tube indicated the presence of reducing sugars.

Molish's Test

Two millilitres of the crude extract were mixed with 2 mL of Molish's reagent, and the mixture was shaken. Approximately 2 mL of concentrated H_2SO_4 were poured carefully along the side of the test tube. The formation of a violet ring at the interphase indicated the presence of carbohydrates.

Benedict's Test

Two millilitres of the crude extract were mixed with 2 mL of Benedict's reagent. The formation of a reddish-brown precipitate after boiling indicated the presence of reducing sugars.

Iodine Test

The crude extract was mixed with 2 mL of iodine solution. The appearance of a dark blue or purple colour indicated the presence of starch.

Anti-cancer and Cytotoxicity Tests

Anti-cancer and cytotoxicity tests were determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay as described by Mosmann [11]. The assay plate was analysed by a microplate reader at 570 nm. The anti-cancer test was carried out on HepG2 cells (Hepatocellular carcinoma cell line) whereas the cytotoxicity test was carried out on Vero cells (African Monkey Kidney normal cell line). Absorbance was measured and expressed as a percentage of the control.

RESULTS AND DISCUSSION

Phytochemical Screening of the Rice Plant Extract

Plant growth regulators (PGRs) play an important role in plant growth and development. Cytokinins such as 6-benzylamino purine (BAP) are among the most widely-used PGRs in plant tissue culture for shoot induction. According to Saleh, the optimum concentration of BAP for maximum shoot induction of *Oryza sativa* L. Cv. MRQ 74 was 0.1 mg/L [12]. Therefore, the same concentration of BAP was added into the culture media in the current study. Figure 1 illustrates 6-week-old aseptic seedlings used as explant sources; whereas Figure 2 shows 5-week-old plantlets used as crude extract sources.

The results of the phytochemical analysis of the crude extracts derived from the stems and leaves of *Oryza sativa* L. Cv. MRQ 74 grown on MS media, with and without the presence of BAP, are provided in Table 1. Based on the results obtained, both crude extracts showed positive results for saponins, terpenoids and resins. The saponins test produced froth when the crude extracts were mixed with distilled water. The occurrence of a greyish colour indicated the presence of terpenoids when the crude extracts were added to concentrated sulphuric acid and then heated for 2 minutes. A green colour was observed after copper acetate was mixed with the crude extracts, suggesting the presence of resins in the

Figure 1. Six-week-old seedlings grown on hormone-free MS media

Figure 2. Five-week-old plantlets grown on MS media supplemented with 0.1 mg/L BAP

Table 1. Phytochemical constituents of *Oryza sativa* L. Cv. MRQ 74

Phytochemical analysis	Crude extracts without BAP	Crude extracts with BAP
Flavonoids	-	-
Alkaloids	-	-
Saponins	+	+
Tannins	-	-
Terpenoids	+	+
Resins	+	+
Carbohydrate properties	**Crude extracts without BAP**	**Crude extracts with BAP**
Reducing sugar (Fehling's test)	+	+
Reducing sugar (Benedict's test)	+	-
Carbohydrates	-	-
Starch	-	-

Note: '+' present and '-' absent.

sample. Both samples of the crude extracts were positive for reducing sugars, but gave negative results for carbohydrates and starch.

Saponin is a natural surface-active glycoside produced by plants, which consists of a sugar moiety linked to a hydrophobic aglycone called sapogenin (a steroid or triterpene). This compound has significant effects on lipid metabolism, which in turn can be used as a treatment for obesity [13]. Furthermore, this compound also possesses anti-inflammatory, immunostimulant, hypocholesterolaemic, hypoglycaemic, antifungal and cytotoxic properties.

'Terpenoid' indicates the presence of terpene constituents. Terpenes are polymers of five carbon hydrocarbon isoprene and heterogenous lipids found in all living organisms and natural products [14]. Since ancient times, terpenes have been used medicinally, e.g., eucalyptus oil and cloverleaf oil in dentistry. They help in stimulating the secretion of mucus, and hence, act as expectorants [15-16].

Resins are a secondary metabolic by-product of plants produced by normal plant tissue or when certain parts of the plant, such as bark or wood, are injured. On the other hand, resins have been used as varnishes or protective coatings since ancient times. Natural resins such as rosin, damar, copal, sandarac, amber, and manila are particularly important as furniture coatings [17].

All monosaccharides and some disaccharides and polysaccharides are reducing sugars, which have a free aldehyde or ketone group. Positive results from the Fehling's and Benedict's tests are due to the presence of an aldehyde in the sample. A negative result for Benedict's test, in contrast, may probably be due to the occurrence of a ketose in rice.

Biological Activities of the Rice Plant Extract

A bioassay is a biological standardisation related to scientific experiments that utilise living things to test the toxicity of chemicals, hence, it gives valuable information about the potency of biological products [18-19]. Moreover, it is also a guided experiment to show the substance's effect on living organisms and is necessary for the development of new drugs [20]. Therefore, bioassay experiments for anti-cancer and cytotoxicity were carried out on crude extracts of the *in vitro* grown rice plant (*Oryza sativa* L. Cv. MRQ 74).

Anti-cancer Test on Hepatocellular Carcinoma Cell Line (HepG2 Cell)

Tables 2 and 3 depict the results of the anti-cancer test on HepG2 cells associated with cell viability as a percent of control crude extracts and crude extracts containing BAP, respectively. Concentrations at which 50% cell death (IC_{50}) were recorded in the control crude extract samples were in the range of 500 µg/mL and 1000 µg/mL, as shown in Table 2. In contrast, Table 3 shows that the BAP crude extract samples had IC_{50} values ranging from 250 µg/mL to 500 µg/mL. The results also suggest that the crude extracts derived from rice plants that were cultured on MS media containing BAP may act as a better anti-cancer agent, with lower concentrations needed to inhibit cancerous cell growth. Incidentally, Ramakrishna et al. found that a rice callus suspension culture had the ability to kill more than 95% of cancer cells without any significant effect on the growth of normal cells [21].

Table 2. Cell viability (%) against the concentration of the control crude extract of *in vitro* grown rice plants

Control crude extract (µg/mL)	% of Cell Viability
0.12	118.90
0.24	107.45
0.49	105.97
0.98	113.53
1.95	110.96
3.91	112.49
7.81	123.18
15.63	114.03
31.25	116.60
62.50	105.48
125.00	98.58
250.00	90.30
***500.00**	**71.78**
***1000.00**	**18.14**

Note: *****Best concentration range of IC_{50}

Table 3. Cell viability (%) against the concentration of the crude extract of *in vitro* grown rice plants with BAP

Crude extract with BAP (µg/mL)	% of Cell Viability
0.12	109.62
0.24	88.74
0.49	94.81
0.98	93.94
1.95	101.92
3.91	105.68
7.81	97.74
15.63	95.19
31.25	92.16
62.50	91.58
125.00	80.38
***250.00**	**86.87**
***500.00**	**44.44**
1000.00	9.04

Note: *****Best concentration range of IC_{50}

Cytotoxicity Test on African Monkey Kidney Normal Cell Line (Vero Cells)

Results of the cytotoxicity tests performed on Vero Cells in relation to the percent of cell viability versus the concentrations of the control and BAP crude extracts of the *in vitro* grown rice plant samples are provided in Tables 4 and 5, respectively. The results obtained show that the IC_{50} values of the control crude extract samples were at 312.50 µg/mL and 625.00 µg/mL (Table 4). On the contrary, the IC_{50} values of BAP crude extract samples ranged from 625.00 µg/mL to 1250 µg/mL (Table 5). These results show that the BAP crude extracts reacted better compared to the control crude extracts. In addition, the BAP crude extracts had lower toxicity to normal cells even at high concentrations. The results from the present study were in line with Norhaizan et al. who reported that the PA extracted from rice bran inhibited the growth of ovary, breast and liver cancer cells but exhibited no sensitivity towards the normal cell line [22].

Table 4. Percent of cell viability versus concentration of the control crude extract of *in vitro* grown rice plants

Control crude extract (µg/mL)	% of Cell Viability
1.22	129.84
2.44	130.49
4.88	130.12
9.77	127.27
19.53	124.24
29.06	121.21
78.13	120.48
156.25	110.84
***312.50**	**93.20**
***625.00**	**24.61**
1250.00	18.18
2500.00	33.70
5000.00	45.45
10000.00	75.57

Note: *****Best concentration range of IC_{50}

Table 5. Percent of cell viability versus concentration of the crude extract of *in vitro* grown rice plants with BAP

Crude extract with BAP (µg/mL)	% of Cell Viability
1.22	128.03
2.44	128.73
4.88	127.50
9.77	120.63
19.53	117.63
29.06	114.55
78.13	108.30
156.25	109.26
312.50	98.42
***625.00**	**83.00**
***1250.00**	**30.31**
2500.00	20.80
5000.00	30.49
10000.00	78.95

Note: *****Best concentration range of IC_{50}

CONCLUSION

The present study revealed that all crude extracts of the *in vitro* cultured rice (*Oryza sativa* L. Cv. MRQ 74) contained saponins, terpenes, resins and reducing sugars. The crude extracts derived from the *in vitro* grown rice on MS media containing BAP showed better results than the control crude extracts in the cytotoxicity and anti-cancer tests. Therefore, further research needs to be carried out to identify the compounds in this economically important plant that have the potential to be used as anti-cancer agents.

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The authors declare that they have no conflict of interest.

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