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Research Article

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In Vitro Antioxidant and Cytotoxic Activity of Aum-Ma-Rit Recipe, A Traditional Herbal Drug for Treatment of Gi Cancer

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Abstract: Aum-Ma-Rit recipe (R-AMR), a traditional Thai drug, has been recorded for treatment of GI disorders for several decades. It has been clinically applied as a complementary treatment for taking care of colon cancer patients. However, there are no scientific studies to confirm its traditional use. Therefore, this study was aimed to primarily screen biological properties of R-AMR on anti-oxidation as well as anticancer against HT-29 human colon cancer. The recipe with and without tamarind as an aqueous adjuvant was extracted using two types of solvents, water and ethanol. Total phenolic and total flavonoid contents of all tested extracts were evaluated. LC-MS/MS was also performed. DPPH, ABTS, FRAP, and ORAC scavenging assays were used to measure an *in vitro* antioxidant activity and the property of extracts on killing HT-29 human colon cancer cells was investigated using MTT assay. The result revealed that all sample extracts were composed of a huge source of phenolic and flavonoid. R-AMR2.2 possessed an intense antioxidant property against DPPH (17.45 ± 0.3 µg/ml), ABTS (4.04 ±0.01 µg/ml), FRAP (6859.26 ± 0.00 µM of $FeSO_4$ equivalent/mg of extract), ORAC (334.06 ± 18.52 µM of trolox equivalent/mg of extract), and also inhibiting lipid peroxidation (88.78 ± 1.11 %). MTT assay revealed that extracts have no cytotoxic effects on HT-29 human colon cancer cells. R-AMR had a strong ability on anti-oxidation even if it had less cytotoxic effects on HT-29. Therefore, the current study exhibited that the recipe may act as a chemopreventive agent for taking care of cancer patients.

Keywords: Aum-Ma-Rit recipe, Traditional Thai recipe, Antioxidant activities, Anti-cancers, Chemopreventive agent

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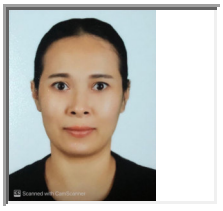

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1. INTRODUCTION

Cancer is the major public health problem which is considered the second leading cause of death globally. According to a WHO report, it is estimated that about 70% of cancer deaths occur in low- and middle-income countries. [1] In 2018, colorectal cancer is categorized as the second cancer problem leading to mortality all over the world.[1] It has been often found in over 60 years old patients. However, it can currently be diagnosed at various ages especially working age. Its pathogenesis happens through complex multistep carcinogenesis. Oxidative stress is well known as a biochemical condition characterized as imbalanced between high levels of toxic reactive substances and the antioxidant defense mechanisms. [2] Excessive free radicals can lead to DNA damage, malignant progression, cell apoptosis, cell metastasis, as well as resistance to cancer treatment. [2] Some conventional treatments of colon cancer such as chemotherapy as well as radiotherapy can generate free radical production causing various adverse effects in patients during cancer therapy. [3] During the past decades, the growing interest in medicinal plants has become more significant, beneficial, and useful for cancer treatment because they are rich in various essential phytochemical compounds.[4] As traditional Thai medicine, several medicinal plants have been applied as a recipe for cancer treatment and taking care of quality of life in patients. Nowadays, some of them were brought to evaluate biological activities especially anticancer activity such as of Tri-Pha-La [5] and Benja Amarit. [6-7] Aum-Ma-Rit recipe (R-AMR) has been found in Thad-Ban-Jhob Thai scripture that is a standard traditional Thai medicine textbook. It has been recorded for the treatment of GI tract diseases that causes the abnormal stool (Health Technical Office, Ministry of Public Health,1998) and now it has been preliminarily applied for taking care of the quality of life in cancer patients, especially colon cancer (J Chokpaisarn, 2019, unpublished clinical data). It contains 18 herbal plants and one herbal aqueous adjuvant as explained in Table 1. Until now, there has been no scientific evidence reporting on its biological properties. However, each medicinal plant belonging to the recipe has extensively contributed to various biological properties such as anti-oxidation [8-11], anti-inflammation [12-14], as well as anti-cancer. [15-16] Therefore, this study was aimed to primarily screen biological properties of R-AMR on anti-oxidation as well as anticancer against HT-29 human colon cancer to prove its traditional use. Moreover, its phytochemical profile has also been investigated.

2. MATERIALS AND METHODS

2.1 Medicinal plant materials

All of the Thai medicinal plants in Aum-Ma-Rit (R-AMR) remedy as described in Table 1 were bought from Fha Sen Osot store, a famous traditional herbal store, at Songkla, Thailand. Plants were identified by a botanist and then deposited as reference voucher specimens at the Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand (HRMAR001- 018). All plants were washed and dried in a hot air oven at 60°C for 24 h. They were partially pulverized into a fine powder for the extraction. According to the original Thai scripture, tamarind (150 g) has been used as an aqueous adjuvant and slightly added to make the powdered recipe be solidified. Therefore, this study has prepared the recipe powder in two types, R-AMR with tamarind and R-AMR without tamarind. Then, the powder of each medicinal plant was finely mixed for the total 300 g of powder formula and the powder formula without tamarind for the total 150 g of powder extract as shown in Table 1.

Table 1 The scientific name, part of used and quantity of use of medicinal plants in R-AMR formula.

Scientific name	Part of used	Weight (g)
Tamarindus indica Linn.	Fruits	150
Rheum officinale Baill, *Rheum palmatum* L, *Rheum tanguticum* (Maxim. Ex Regel) Maxim.ex Balf)	Rhizomes	45
Aloe vera (L.) Burm.f.	Latex	15
Ferula assa-foetida L	Oleo-gum-resin	15
Aegle marmelos (L.) Correa	Fruit	15
Acorus calamus L.	Rhizome	15
Baliospermum montanum Muell.A	Root	3.75
Citrus hystrix DC.	Fruit peel	3.75
Terminalia chebula Retz.	Fruits	3.75
Terminalia bellirica (Gaertn.) Roxb.	Fruits	3.75
Piper nigrum Linn.	Fruits	3.75
Piper retrofractum Vahl	Fruits	3.75
Zingiber officinale Roscoe.	Rhizome	3.75
Nigella sativa L	Seed	3.75
Lepidium sativum L.	Seed	3.75
Cuminum cyminum L	Seed	3.75
Foeniculum vulgare Miller subsp. var. vulgare	Seed	3.75
Anethum graveolens L	Seed	3.75

2.2 Medicinal plants extract preparation

Both herbal powders were extracted with two types of solvents which were sterile distilled water by boiling at 100°C for 15 minutes and 95% Ethanol (EtOH) for seven days at room temperature. After filtration through a Whatman No.1 filter paper, the aqueous and ethanolic herbal solution was evaporated to dryness using a freeze dryer and a rotary evaporator, respectively. Watery extracts with tamarind (R-AMR1.1), without tamarind (R-AMR1.2), ethanolic extract with tamarind (R-AMR2.1), and without tamarind (R-AMR2.2) were stored in a sterile dark bottle at -20°C and dissolved in 10% dimethyl sulfoxide (DMSO) before use. Yields (%; w/w) of each extract were calculated as the ratio of the weight of the extract to the weight of the raw recipe powder.

2.3 Phytochemical constituent determination

2.3.1 Total phenolic contents

Total phenolic contents (TPC) were performed using Folin-Ciocalteu's reagent assay with slight modification from Kaisoon et al., 2011. Briefly, a 150 µl of Folin-Ciocalteu's reagent diluted in distilled water was mixed with an aliquot of 10 µl of crude extracts at a concentration of 1mg/ml. Then, 50 µl of 10% Na_2CO_3 was added, and the absorbance was detected at 765 nm using a UV-visible spectrophotometer. Gallic acid was used as a standard curve and the result was expressed as gallic acid equivalents (mg of GA/g of extract). [17]

2.3.2 Total flavonoid contents

Total flavonoid contents (TFC) were determined by aluminum chloride colorimetric assay modified from Huang et al., 2018. [18] A 10 µl of 10 % $AlCl_3$ solution which was prepared by dissolving one g of $AlCl_3$ in 10 ml of distilled water was mixed with an aliquot of 10 µl of CH_3COOK. Then, 50 µl of crude extracts at a concentration of 1 mg/ml and 150 µl of methanol were added. The final volume of the reaction was adjusted with 30 µl of distilled water. The absorbance was detected at 415 nm using a UV-visible spectrophotometer. Quercetin was used as a standard curve and the result was expressed as mg of quercetin/g of extract.

2.3.3 LC-MS/MS analysis

Liquid chromatography-mass spectrometry was performed at the Office of Scientific Instrument and Testing (OSIT), Prince of Songkla University according to the protocol of Qiao et al., 2018 with a slight modification. Briefly, liquid chromatography (Agilent UHPLC 1290 infinity II) coupled with quadrupole time of flight (Q-TOF 6540 accurate mass) was used for phytochemical constituent analysis with column (ZORBAX EclipsePlusC18 RRHD 1.8um, 2.1x150 mm.). A mobile phase condition was used as a gradient which consists of 0.1% formic acid, water (A) and 0.1% formic acid, 100% acetonitrile (B) (0-3.50 min, 5% B; 3.50-13.50 min, 25% B; 13.50-18 min, 35% B; 18-27 min, 45% B; 27-38 min, 38-42 min, 100% B; 42-45 min, 5% B) with flow rate at 0.2 ml/min and 5 ml of injection volume. The mass spectrometric condition; flow rate 13 l/min, 350°C, nebulizer 35 psig, and range measured 50-1500 amu in negative mode. [19] For the detection, the spectrum of compounds found in all extracts was identified based on the National Institute Standard and Technology (NIST).

2.4 In Vitro antioxidant activities

2.4.1 DPPH and ABTS radical scavenging activity

Antioxidant activity of 4 formula extract was evaluated using DPPH radical scavenging activity according to Chanthasri *et al*., 2018. [20] Briefly, an aliquot of 20 µl of the extract solution at final concentrations between 0.98-500 µg/ml (2-fold dilution) were mixed with 180 µl of DPPH-purple solution at a concentration of 80 µM. The mixture was incubated at room temperature for 30 minutes, and the absorbance of the solution was measured at 520 nm using a UV-Vis spectrophotometer. A result was calculated and expressed as IC_{50}. For ABTS radical scavenging activity, ABTS solution was prepared by mixing 2 mM of ABTS solution with 2.45 mM potassium persulfate solution and incubated in the dark at room temperature for 16 h. Then, the ABTS solution was diluted with distilled water to obtain the absorbance of 0.7 ± 0.02 at 734 nm. A volume of 270 µl of ABTS solution was mixed with an aliquot of 30 µl of R-AMR01 or R-AMR02 solution at concentrations between 0.49-500 µg/ml (2-fold dilution). The mixture was incubated in the dark at room temperature for 6 minutes. The absorbance of the mixture was measured at 734 nm, and the result was reported as IC_{50}. Trolox and gallic acid were used as a standard antioxidant activity for both experiments. The free radical scavenging activity was calculated according to the following equation;

$$\% \text{ Inhibition} = [A_{Control} - (A_{Sample} - A_{Blank})] / A_{Control} \times 100$$

Where $A_{Control}$ is the absorbance value of DPPH solution, A_{Sample} is the absorbance value of the sample mixed with DPPH solution and A_{Blank} is the absorbance value of the sample without DPPH solution. The percentage of free radical scavenging was plotted against the concentration of the sample and was calculated from the obtained linear equation for IC_{50} value.

2.4.2 Ferric-reducing antioxidant power

Ferric-reducing antioxidant power (FRAP) was assessed to measure antioxidant activity by a colorimetric reaction of reduction of Ferric (Fe^{3+}). Briefly, a volume of 30 µl of R-AMR extract solution at a final concentration of 5 µg/ml was added to 270 µl of FRAP solution. The mixture was incubated in the dark at room temperature for 30 minutes. After the incubation period, the reaction was measured at the absorbance of 596 nm using an UV-visible spectrophotometer. The result was compared with the ferrous sulfate standard curve and performed as a FRAP value in µM equivalent of FeSO4/mg of extract using a linear equation. [21]

2.4.3 Oxygen radical absorbance capacity

The antioxidant capacity of the extract was determined using the oxygen radical absorbance capacity (ORAC) assay according to a previous report. [22] The assay was used to examine the activity of extracts on fluorescein in the presence of peroxyl radicals generated by AAPH. Briefly, fluorescein sodium salt solution was prepared by dissolving 0.15 mg

fluorescein sodium salt in 75 mM phosphate buffer saline. R-AMR extracts were serially diluted to obtain the concentration at 0.078-5 µg/ml and then mixed with prepared fluorescein sodium salt solution (25 µ l) in 96-well polypropylene fluorescence plates. Plates were incubated at 37°C for 30 minutes. The AAPH solution at a concentration of 153 mM was added in a volume of 25 µl. After the addition of AAPH, the plate was immediately measured at 37°C every 2-minute intervals for 19 minutes at the excitation wavelength of 485 nm and an emission wavelength of 590 nm. Assays conducted with Trolox and phosphate buffer saline were used as a standard and blank control, respectively. Results were calculated by taking the areas under curves (AUC) and expressed as an ORAC unit.

2.4.4 Lipid peroxidation (LPO)

LPO was evaluated by the measurement of malondialdehyde content (MDA) in the form of thiobarbituric acid-reacting substances (TBARS) according to a previous report with slight modification. [23] Briefly, an aliquot (50 µl) of egg yolk in potassium chloride solution at a concentration of 12.5 mg/ml was gently mixed with R-AMR extract solution at concentrations of 0.13, 0.5, and 2 mg/ml. Then, 20% acetic acid (150 µl) and 0.8% thiobarbituric acid (150 µl) in 1% sodium dodecyl sulfate were subsequently added. Then, sterile distilled water was finally filled to adjust the volume of the mixture to 400 µl and heated in a boiling water bath at 95°C for an hour. After cooling, the reaction mixture was measured at 532 nm and reported as a percentage inhibition using OD value to calculate. Butylated hydroxytoluene (BHT) was used as a standard control.

2.5 In vitro cancer cytotoxicity

2.5.1 Maintenance of HT-29 cell lines for cytotoxicity assay

HT29 cell in this study was obtained from National Cancer Institute, Bangkok, Thailand. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in condition of 5% CO_2.

2.5.2 Treatment of cell with R-AMR extracts

A cellular suspension with 3 × 10^3 cells per well was seeded (100 µ L) in 96-well flat-bottom plates and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. R-AMR extracts were dissolved in sterile distilled water or 0.1% DMSO and subsequently diluted in the culture medium to obtain concentrations ranging from 3 – 1,000 µg/ml. After 24 h of the incubation time, the culture medium was carefully removed and replaced with a fresh medium containing R-AMR extract solution. Plates were further incubated at 37°C in a 5% CO_2 atmosphere for 72 h.

2.5.3 Cytotoxicity test of R-AMR extracts

Cytotoxicity of R-AMR extracts was evaluated against HT-29 cell lines by MTT tetrazolium salt assay. After the incubation period, cells were allowed to react with 5 µl MTT solution (5 mg/mL in PBS). At the end of the incubation period, the medium was removed and replaced with 100 µ L of DMSO to dissolve the MTT formazan crystals. The absorbance was measured at 550 nm, with 595 nm as the reference wavelength. The resulting value was calculated and indicated as IC_{50}.

2.6 Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) by SPSS version 20and was considered a significant difference using Tukey's post hoc test at a significant level of 95%.

3. RESULTS

3.1 Phytochemical screening of R-AMR extracts

Herbal plants are a major source of phenolic and flavonoid contents that relate to several potential biological properties including anti-inflammatory, anti-oxidant, as well as anticancer activities. Therefore, total phenolic and total flavonoid compounds were preliminarily determined. The results found that all sample extracts were composed of a huge source of phenolic and flavonoid (Table 2). R-AMR2.2 highly consisted of both phenolic and flavonoid at 733.76 ± 22.92 mg of gallic acid/g of extract and 114.61± 6.88 mg of quercetin/g extract, respectively. Compared between the watery and ethanolic extracts, it was obviously shown that both phenolic and flavonoid could be more extracted by ethanol fermentation than watery boiling. Notably, the extracts without tamarind (R-AMR1.2 and R-AMR2.2) seemed to have a higher amount of both total phenolic and flavonoid contents when compared with the extracts composed of tamarind (Table 2).

***Table 2* Total phenolic and total flavonoid contents of R-AMR extracts.**

Sample	% Yield	Total phenolic contents (mg of gallic acid/g Extract)	Total flavonoid contents (mg of quercetin/g Extract)
R-AMR1.1	12.99	157.10 ± 3.23^{d}	28.36 ± 4.34^{d}
R-AMR1.2	6.91	328.38 ± 8.32^{c}	53.51 ± 1.31^{c}
R-AMR2.1	16.29	438.29 ± 7.81^{b}	60.54 ± 0.90^{b}
R-AMR2.2	20.47	733.76 ± 22.92^{a}	114.61 ± 6.88^{a}

Each value in the table is represented as mean ± SE (n=3). Superscript letters with different letters in the same column indicate a significant difference (p≤0.05) analyzed by Tukey's post hoc test.

The phytochemical profile of each extract was determined by LC-MS/MS in negative mode and then phenolic and flavonoid compounds were identified based on a reliable database. Figure 1 and Figure 2 present LC-MS/MS chromatography of watery and ethanolic R-AMR extracts, respectively. Chromatograms were amplified by the name, molecular formula, and molecular weight in Table 3. There were 22 and 26 identified compounds found in R-AMR1.1 and R-AMR1.2, respectively while R-AMR2.1 and R-AMR2.2 possessed 26 and 27 compounds, respectively. Based on the PubChem and

Human metabolome database, almost all compounds were classified as phenolic and flavonoids (Table S1 of Supplementary data). Out of 48 compounds, R-AMR1.1 was identified for 10 phenolic and nine flavonoid compounds, while R-AMR1.2 was 13 and six compounds identified as phenolic and flavonoids, respectively. For ethanolic extracts, there were 11 phenolics and 10 flavonoids identified in R-AMR2.1, while R-AMR2.2 classified as phenolic and flavonoid for 10 and eight compounds, respectively. Five compounds including 3-Glucogallic acid, Macrocarposide, Catechin 4'-methyl ether, Afzelechin 7-apioside, and 2''-o-p-Coumaroylaloesin was found in all of four formula extracts and there were six compounds, 11-Methylgerberinol, Ampelopsin D, 2'',4'',6''-Triacetylglycitin, 2'-Methoxyformonetin, Artonol B, and cis-p-Coumaroylcorosolic acid, that have been discovered only R-AMR2.2.

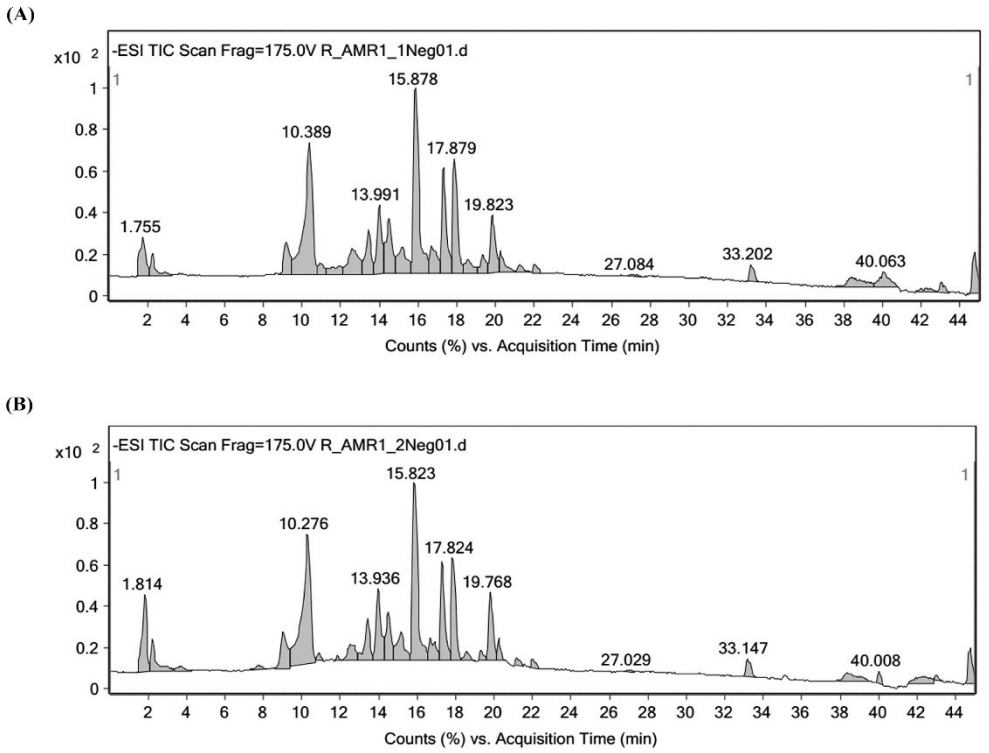

Fig 1 Phytochemical profile of watery R-AMR1.1 (A), R-AMR1.2 (B) extracts by LC-QTOF-MS/MS

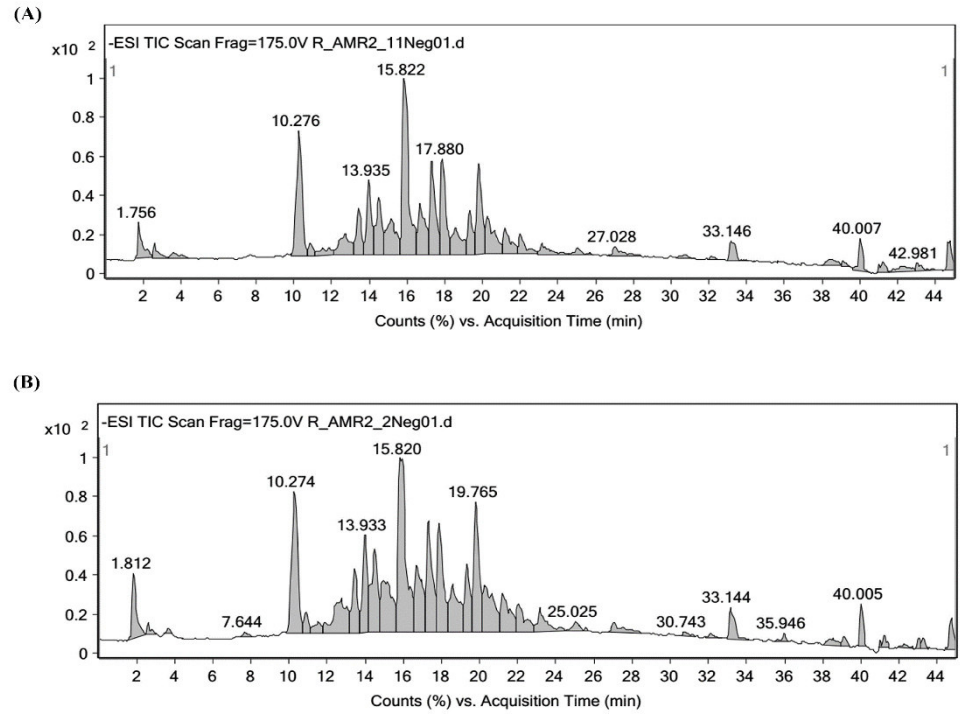

Fig 2 Phytochemical profile of ethanolic R-AMR2.1 (A) and R-AMR2.2 (B) extracts by LC-QTOF-MS/MS

Table 3 An identified phytochemical compound of R-AMR extracts detected by LC-MS.

Sample	No.	R_t	Compound name	Molecular formula	Molecular weight
R-AMR1.1	1	1.949	Tartaric acid	$C_4H_6O_6$	150.014
	2	2.26	(+)-Chebulic acid	$C_{14}H_{12}O_{11}$	356.037
	3	2.329	3-Glucogallic acid	$C_{13}H_{16}O_{10}$	332.074
	4	9.26	2'-Oxoaloesol 7-glucoside	$C_{19}H_{22}O_9$	394.1273
	5	10.093	5,7,3',6'-Tetrahydroxy-8, 2'-dimethoxyflavone 6'-glucoside	$C_{23}H_{24}O_{13}$	508.120
	6	10.971	Octanoylglucuronide	$C_{14}H_{24}O_8$	320.147
	7	12.429	Macrocarposide	$C_{21}H_{22}O_{11}$	450.117
	8	12.744	Catechin 4'-methyl ether	$C_{16}H_{16}O_6$	304.0951
	9	12.907	Steganacin	$C_{24}H_{24}O_9$	456.143
	10	13.009	Quercetin Tetramethyl (5,7,3',4') Ether	$C_{19}H_{18}O_7$	358.106
	11	13.951	Afzelechin 7-apioside	$C_{20}H_{22}O_9$	406.127
	12	15.006	Naringenin-4'-O-glucuronide	$C_{21}H_{20}O_{11}$	448.101
	13	15.463	Resveratrol 4'-(2-galloylglucoside)	$C_{27}H_{26}O_{12}$	542.1439
	14	15.835	2"-o-p-Coumaroylaloesin	$C_{28}H_{28}O_{11}$	540.165
	15	17.322	Aloin A	$C_{21}H_{22}O_9$	418.128
	16	19.376	2,8-Dihydroxy-3,4,9,10-tetramethoxypterocarpan	$C_{19}H_{20}O_8$	376.116
	17	19.666	Rhapontigenin	$C_{15}H_{14}O_4$	258.090
	18	19.854	Resveratrol 4'-Methyl Ether	$C_{15}H_{14}O_3$	242.095
	19	20.066	Luteolin 3'-rhamnoside	$C_{21}H_{20}O_{10}$	432.106
	20	20.385	Riboflavin cyclic-4',5'-phosphate	$C_{17}H_{19}N_4O_8P$	438.093
	21	21.267	Chrysophanol 8-(6-galloylglucoside)	$C_{28}H_{24}O_{13}$	568.122
	22	33.176	Apigenin	$C_{15}H_{10}O_5$	270.054
R-AMR1.2	1	1.788	Gulonic acid	$C_6H_{12}O_7$	196.059
	2	1.89	Sucrose	$C_{12}H_{22}O_{11}$	342.116
	3	1.939	Quinic acid	$C_7H_{12}O_6$	192.063
	4	2.065	Malic acid	$C_4H_6O_5$	134.022
	5	2.229	(+)-Chebulic acid	$C_{14}H_{12}O_{11}$	356.039
	6	2.36	3-Glucogallic acid	$C_{13}H_{16}O_{10}$	332.075
	7	7.832	Glucotropaeolin	$C_{14}H_{19}NO_9S_2$	409.051
	8	9.924	5,7,3',6'-Tetrahydroxy-8,2'-dimethoxyflavone 6'-glucoside	$C_{23}H_{24}O_{13}$	508.120
	9	10.936	Octanoylglucuronide	$C_{14}H_{24}O_8$	320.147
	10	12.402	Macrocarposide	$C_{21}H_{22}O_{11}$	450.117
	11	13.435	2'-Oxoaloesol 7-glucoside	$C_{19}H_{22}O_9$	394.128
	12	13.896	Afzelechin 7-apioside	$C_{20}H_{22}O_9$	406.128
	13	14.31	Catechin 4'-methyl ether	$C_{16}H_{16}O_6$	304.095
	14	14.73	Ellagic acid	$C_{14}H_6O_8$	302.007
	15	15.853	Abruquinone C	$C_{19}H_{20}O_8$	376.117
	16	16.102	2"-o-p-Coumaroylaloesin	$C_{28}H_{28}O_{11}$	540.165
	17	16.928	Neohesperidin	$C_{28}H_{34}O_{15}$	610.190
	18	17.323	Aloin A	$C_{21}H_{22}O_9$	418.128
	19	19.668	Rhapontigenin	$C_{15}H_{14}O_4$	258.090
	20	19.801	Piceid	$C_{21}H_{24}O_8$	404.148
	21	19.973	Resveratrol 4'-Methyl Ether	$C_{15}H_{14}O_3$	242.0947
	22	20.321	Riboflavin cyclic-4',5'-phosphate	$C_{17}H_{19}N_4O_8P$	438.0933
	23	20.382	3,7-Dihydroxyflavone	$C_{15}H_{10}O_4$	254.0585
	24	22.02	Physcion	$C_{16}H_{12}O_5$	284.069
	25	33.161	Baicalein	$C_{15}H_{10}O_5$	270.0535
	26	35.165	Kamolonol	$C_{24}H_{30}O_5$	398.2097
R-AMR2.1	1	1.951	Tartaric acid	$C_4H_6O_6$	150.014
	2	2.808	3-Glucogallic acid	$C_{13}H_{16}O_{10}$	332.075
	3	3.788	Gallic acid	$C_7H_6O_5$	170.022
	4	7.431	Glucotropaeolin	$C_{14}H_{19}NO_9S_2$	409.051
	5	12.393	Macrocarposide	$C_{21}H_{22}O_{11}$	450.117

	6	13.435	2'-Oxoaloesol 7-glucoside	$C_{19}H_{22}O_9$	394.128
	7	13.952	Afzelechin 7-apioside	$C_{20}H_{22}O_9$	406.128
	8	14.309	Catechin 4'-methyl ether	$C_{16}H_{16}O_6$	304.095
	9	14.489	2"-o-p-Coumaroylaloesin	$C_{28}H_{28}O_{11}$	540.164
	10	14.738	Ellagic acid	$C_{14}H_6O_8$	302.007
	11	14.844	(-)-Epicatechin 3-O-gallate	$C_{22}H_{18}O_{10}$	442.091
	12	15.044	Piceatannol 4'-galloylglucoside	$C_{27}H_{26}O_{13}$	558.138
	13	15.203	Rhaponticin	$C_{22}H_{26}O_{11}$	466.148
	14	15.855	2,8-Dihydroxy-3,4,9,10-tetramethoxypterocarpan	$C_{19}H_{20}O_8$	376.117
	15	17.323	Aloin A	$C_{21}H_{22}O_9$	418.128
	16	19.647	Rhapontigenin	$C_{15}H_{14}O_4$	258.090
	17	19.741	Asebotin	$C_{22}H_{26}O_{10}$	450.154
	18	20.01	Luteolin 3'-rhamnoside	$C_{21}H_{20}O_{10}$	432.107
	19	21.211	Chrysophanol 8-(6-galloylglucoside)	$C_{28}H_{24}O_{13}$	568.123
	20	22.068	Biochanin A	$C_{16}H_{12}O_5$	284.069
	21	25.05	Madecassic Acid	$C_{30}H_{48}O_6$	504.346
	22	25.728	Broussoflavonol B	$C_{26}H_{28}O_7$	452.184
	23	27.917	Gerberinol	$C_{21}H_{16}O_6$	364.0951
	24	30.759	DL-Kavain	$C_{14}H_{14}O_3$	230.0946
	25	33.16	Apigenin	$C_{15}H_{10}O_5$	270.0541
	26	39.164	16-hydroxy hexadecanoic acid	$C_{16}H_{32}O_3$	272.2357
R-AMR2.2	1	1.892	Sucrose	$C_{12}H_{22}O_{11}$	342.117
	2	1.917	Quinic acid	$C_7H_{12}O_6$	192.064
	3	2.807	3-Glucogallic acid	$C_{13}H_{16}O_{10}$	332.075
	4	3.501	Gallic acid	$C_7H_6O_5$	170.022
	5	7.716	Glucotropaeolin	$C_{14}H_{19}NO_9S_2$	409.051
	6	10.942	Octanoylglucuronide	$C_{14}H_{24}O_8$	320.147
	7	12.408	Macrocarposide	$C_{21}H_{22}O_{11}$	450.117
	8	12.992	Quercetin Tetramethyl (5,7,3',4') Ether	$C_{19}H_{18}O_7$	358.106
	9	13.95	Afzelechin 7-apioside	$C_{20}H_{22}O_9$	406.128
	10	14.307	Catechin 4'-methyl ether	$C_{16}H_{16}O_6$	304.096
	11	14.728	Ellagic acid	$C_{14}H_6O_8$	302.007
	12	14.867	(-)-Epicatechin 3-O-gallate	$C_{22}H_{18}O_{10}$	442.091
	13	15.062	Piceatannol 4'-galloylglucoside	$C_{27}H_{26}O_{13}$	558.138
	14	15.916	2,8-Dihydroxy-3,4,9,10-tetramethoxypterocarpan	$C_{19}H_{20}O_8$	376.117
	15	16.643	2"-o-p-Coumaroylaloesin	$C_{28}H_{28}O_{11}$	540.165
	16	17.289	2",4",6"-Triacetylglycitin	$C_{28}H_{28}O_{13}$	572.154
	17	17.746	2'-Methoxyformonetin	$C_{17}H_{14}O_5$	298.085
	18	19.584	Artonol B	$C_{24}H_{20}O_7$	420.121
	19	19.839	Resveratrol 4'-Methyl Ether	$C_{15}H_{14}O_3$	242.095
	20	21.266	Chrysophanol 8-(6-galloylglucoside)	$C_{28}H_{24}O_{13}$	568.1226
	21	22.589	11-Methylgerberinol	$C_{22}H_{18}O_6$	378.1111
	22	23.553	Ampelopsin D	$C_{28}H_{22}O_6$	454.1425
	23	25.056	Madecassic Acid	$C_{30}H_{48}O_6$	504.3458
	24	27.915	Gerberinol	$C_{21}H_{16}O_6$	364.0952
	25	30.757	DL-Kavain	$C_{14}H_{14}O_3$	230.0945
	26	39.162	16-hydroxy hexadecanoic acid	$C_{16}H_{32}O_3$	272.2359
	27	39.893	cis-p-Coumaroylcorosolic acid	$C_{39}H_{54}O_6$	618.3927

3.2 The ability of extracts on DPPH and ABTS radical scavenging activity

Measurement of antioxidant with DPPH and ABTS is made based on the mixed-mode-based assay. Table 4 revealed an antioxidant activity of all tested extracts against several free radicals. It indicated that all extracts had a potent activity to inhibit DPPH and ABTS radicals with IC_{50} less than 100 µg/ml. Among the tested extracts, R-AMR2.2 possessed the highest activity with IC_{50} value at 17.45±0.3 and 4.04±0.01 µg/ml against DPPH and ABTS radicals, respectively. Moreover, it is shown that the inhibitory activity of all extracts against both DPPH and ABTS radicals was a dose-dependent pattern (Figure 3A, 3C). Noticeably, both watery and ethanol extracts without tamarind as an aqueous adjuvant were able to inhibit DPPH and ABTS radicals better than the extracts having tamarind.

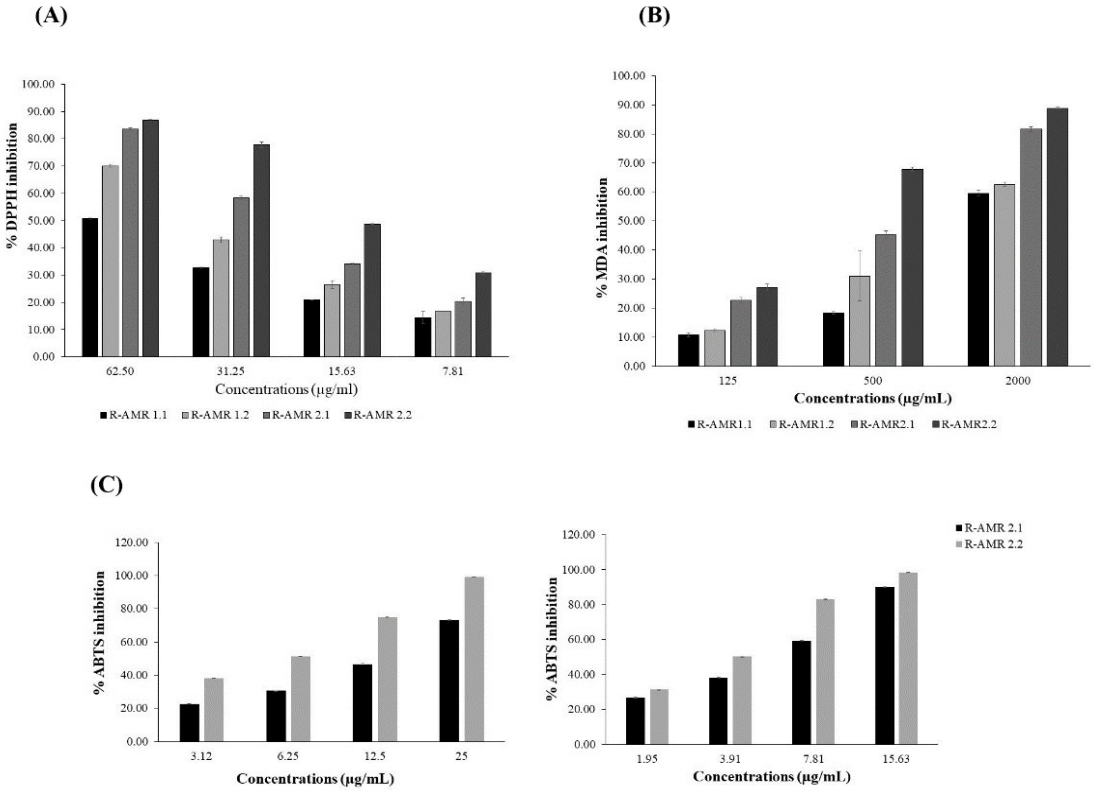

Fig 3 Anti-oxidant activity of R-AMR 1.1, R-AMR 1.2, R-AMR 2.1, and R-AMR 2.2 against DPPH (A), MDA (B), and ABTS (C) radicals.

3.3 The ability of extracts on the ferric-reducing antioxidant power

All extracts were also measured with an antioxidant ability on the ferric (Fe^{3+}) conversion using FRAP assay which was the single electron transfer mechanism-based assay (SET). Table 4 revealed that the greatest antioxidant activity with high FRAP value was recorded for R-AMR2.2 (6,859.26 ± 0.00 µM of $FeSO_4$ equivalent/mg of extract), followed by R-AMR2.1 (4,390.12 ± 17.11 µM of $FeSO_4$ equivalent/mg of extract), R-AMR1.2 (3,116.05 ± 17.11 µM of $FeSO_4$ equivalent/mg of extract), and R-AMR1.1 (2,000 ± 29.63 µM of $FeSO_4$ equivalent/mg of extract).

3.4 Oxygen radical absorbance capacity

The peroxyl radical scavenging activity was performed by Oxygen radical absorbance capacity according to hydrogen atom transfer mechanism-based assay (HAT). The study reveals that R-AMR2.2 exhibited the strong peroxyl radical scavenging properties with the highest ORAC value (334.06 ± 18.52), followed by R-AMR2.1 (96.71 ± 2.33), R-AMR1.2 (62.96 ± 4.33), and R-AMR1.1 (22.45 ± 0.47) (Table 4)

Table 4 Anti-oxidant properties of R-AMR extracts

Samples	IC_{50} (µg/ml)		FRAP value	ORAC value
	DPPH	ABTS	(µM of $FeSO_4$ equivalent/mg of extract)	(µM of trolox equivalent/mg of extract)
R-AMR1.1	72.23 ± 1.47^{f}	14.72 ± 0.04^{f}	2,000 ± 29.63^{d}	22.45 ± 0.47^{d}
R-AMR1.2	40.86 ± 0.54^{e}	6.53 ± 0.02^{e}	3,116.05 ± 17.11^{c}	62.96 ± 4.33^{c}
R-AMR2.1	25.93 ± 0.30^{d}	6.68 ± 0.01^{d}	4,390.12 ± 17.11^{b}	96.71 ± 2.33^{b}
R-AMR2.2	17.45 ± 0.30^{c}	4.04 ±0.01^{c}	6,859.26 ± 0.00^{a}	334.06 ± 18.52^{a}
Trolox	5.06 ± 0.01^{b}	5.15 ± 0.05^{b}	ND	ND
Gallic acid	0.11 ± 0.00^{a}	0.61 ± 0.00^{a}	ND	ND

$^{a-f}$ possessed a significant difference at p≤0.05 (n=3)
ND = Not done

3.5 Inhibition of lipid peroxidation

This study additionally illustrated an antioxidant property using anti-lipid peroxidation ability by reduction of an aldehyde product called Malondialdehyde (MDA) and presented as a percentage of lipid peroxidation inhibition. Among four extracts, R-AMR2.2 revealed the most effective MDA inhibition at all concentrations which extremely inhibit MDA with 88.78 ± 1.11% at the concentration of 2,000 µg/ml. Moreover, this activity was observed in R-AMR2.1, followed by R-AMR1.2, and R-AMR1.1 (Table 5). Figure 3B also revealed that all extracts were dose-dependent to inhibit MDA production, with R-AMR 2.2 having the highest ability at all tested concentrations.

Table 5 Percentage of Inhibition of malondialdehyde (MDA) in lipid peroxidation of R-AMR extract

Samples	Concentration (µg/mL)		
	125	500	2,000
R-AMR1.1	10.80 ± 1.22^{j}	18.31 ± 0.69^{hi}	59.44 ± 0.64^{d}
R-AMR1.2	12.34 ± 0.77^{ij}	31.02 ± 8.63^{f}	62.63 ± 0.51^{cd}
R-AMR2.1	22.7 ± 0.83^{gh}	45.25 ± 1.22^{e}	81.6 ± 0.83^{b}
R-AMR2.2	27.06 ± 0.41^{fg}	67.76 ± 0.71^{c}	88.78 ± 1.11^{a}
BHT	ND	82.43 ± 0.49^{ab}	ND

$^{a-j}$ possessed a significant difference at p≤0.05 (n=3)
ND = Not done

3.6 Cytotoxicity on HT-29 colon cancer cells evaluation

To detect the property of R-AMR extracts on HT-29 human colon cancer cell toxicity, MTT colorimetric assay was performed. The results demonstrated that all extracts were considered inactive against HT-29 human colon cancer cells with IC_{50} value higher than 30 µg/ml after 72 h exposure according to the criteria of National Cancer Institute cancer, USA (Figure 3). It was found that the highest concentration 1,000 µg/mL of R-AMR 1.1, R-AMR 1.2, R-AMR 2.1, and R-AMR 2.2 extracts possessed 83.63%, 84.16%, 29.28%, and 46.96% of cell viability, respectively (Figure 4). Figure 5 also supported that there were not many changes in the cell morphological characters after treatment with all extracts.

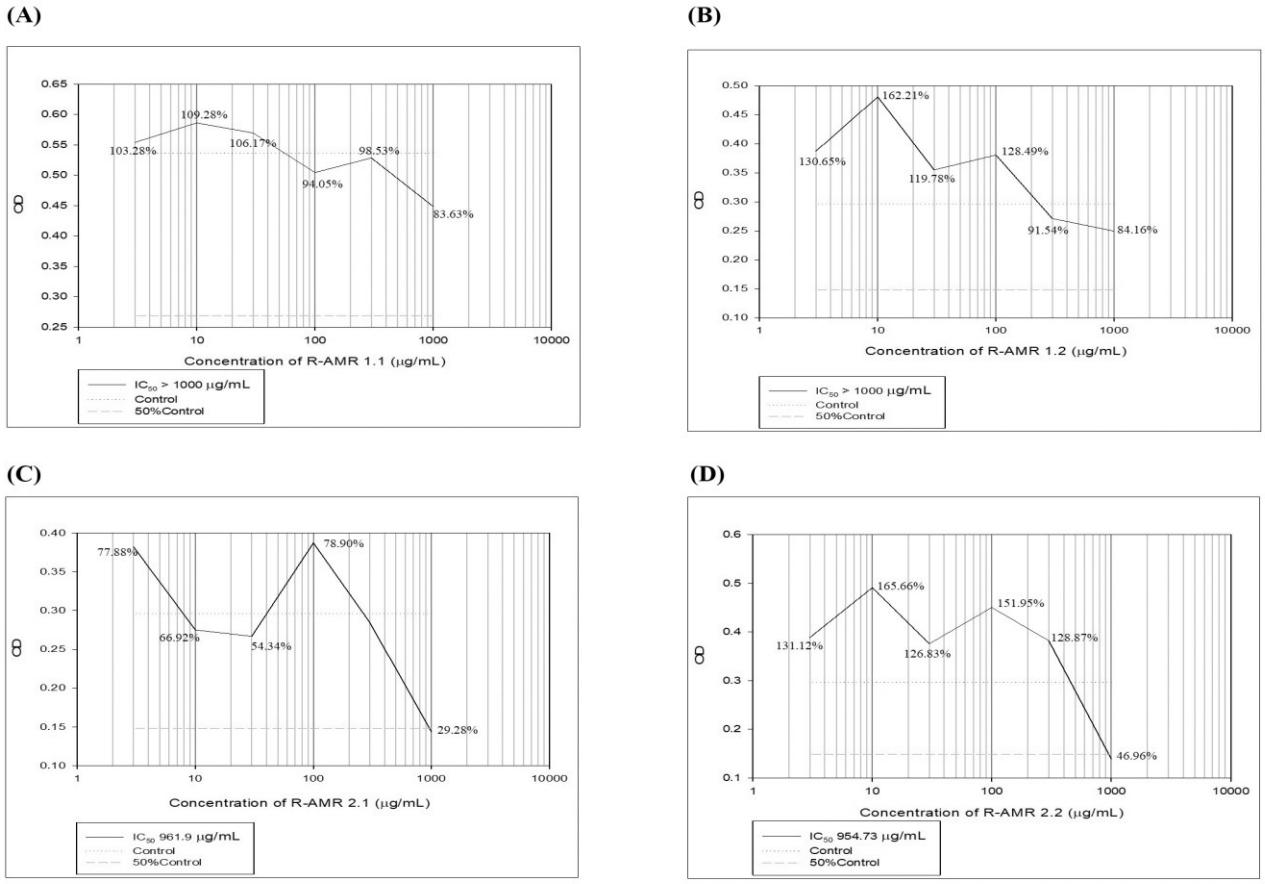

Fig 4 Cell viability of HT-29 human colon cancer cells after treatment with R-AMR 1.1 (A), R-AMR 1.2 (B), R-AMR 2.1 (C), and R-AMR 2.2 (D) at several concentrations

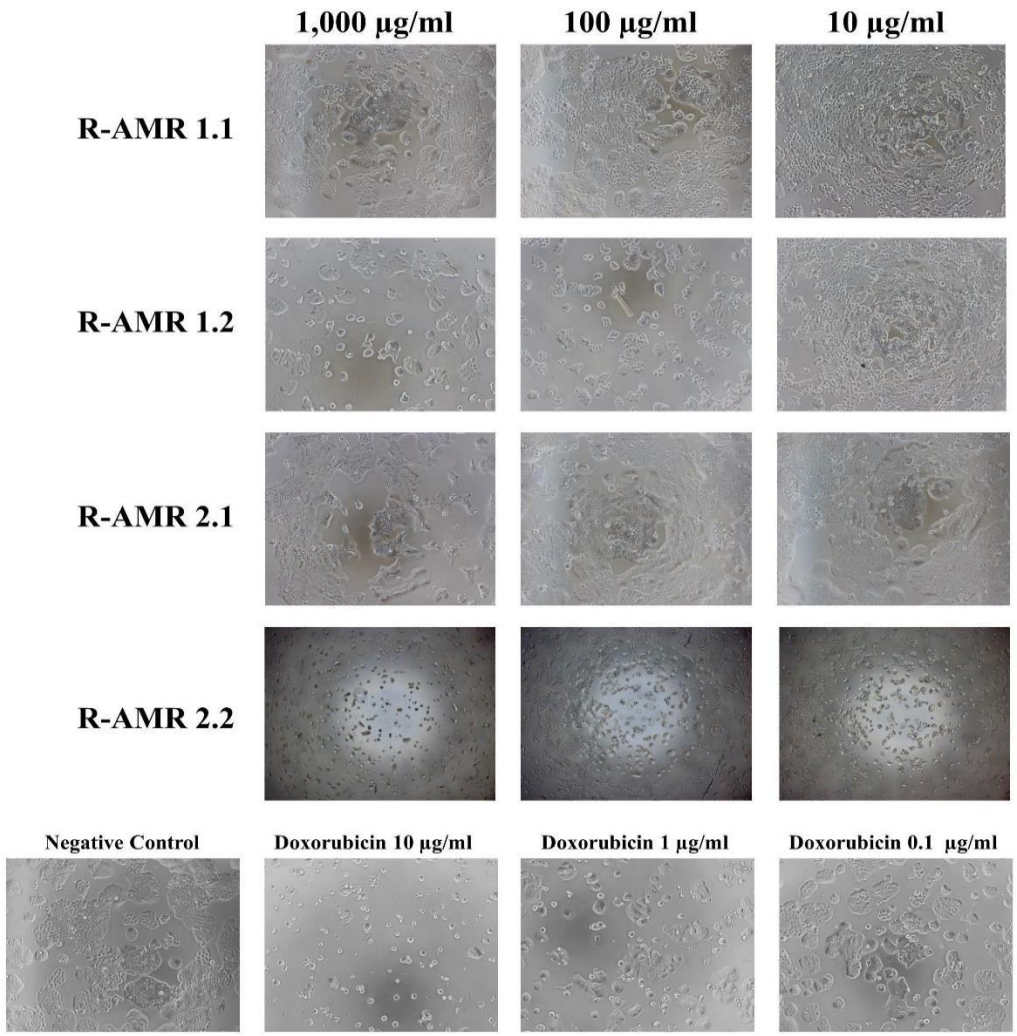

Fig 5 Morphology of HT-29 human colon cancer cells after treatment with R-AMR 1.1, R-AMR 1.2, R-AMR 2.1, R-AMR 2.2 extracts, and Doxorubicin as a positive control at different concentrations for 72 h.

4. DISCUSSION

This current study was the first report that investigated the phytochemical profile as well as biological activities of Aum-Ma-Rit recipe (R-AMR), a traditional Thai drug for the treatment of lower GI tract disorders especially GI cancers, on anti-oxidation and cytotoxic effects on colon cancer cells. The results revealed that Aum-Ma-Rit recipe, especially Aum-Ma-Rit ethanolic extract without tamarind (R-AMR2.2) as an aqueous adjuvant, possessed a remarkable ability to inhibit free radicals in *in vitro* experiment. Moreover, it was also composed of a large amount of total phenolic and total flavonoid contents which related to its antioxidant activity. Even R-AMR had no direct effects on killing HT-29 human colon cancer cells as expected. However, the activity for cancer treatment in patients might be from other biological properties such as an anti-oxidant activity. Besides, it cannot be definitely inferred a non-cytotoxicity of the recipe on the other cancer cell lines because HT-29 cell is the only used cell in our study. Therefore, it might have had a cytotoxic effect on the other cancer cells. Oxidative stress, mainly contributing to the generation of reactive oxygen species (ROS), has been implicated in the pathogenesis of several diseases, especially cancers. Excessive ROS can lead to DNA damage, malignant progression, cell apoptosis, cell metastasis, as well as resistance to cancer treatment. [2] Moreover, it is reported that high ROS levels may potently urge the activation of HIF-1α (hypoxia inducible factor 1α) leading to an increase of VEGF production which is the major cytokine responsible for the process of angiogenesis. [2, 24-25] Our current report demonstrated that R-AMR could inhibit several free radicals *in vitro* experiments potently even they had no effects on cancer cells. Compared with the watery extracts, the ethanol was obviously observed to have a competent ability to inhibit DPPH, ABTS, ferric ion, and peroxyl radicals. Moreover, they were also involved in the suppression of MDA production, leading to the inhibition of LPO. A strong notable antioxidant activity of extracts was correlated with their phenolic and flavonoid contents. R-AMR2.2 was considered to possess the strongest antioxidant property associated with its high amount of phenolic and flavonoid compounds. Numerous reports have revealed that phenolics and flavonoids are the naturally major phytochemical compounds involved in several biological activities, especially an anti-oxidant activity. [26] A previous report elucidated that the ethanolic extract of *Rheum* rhizome, the main medicinal plant of the recipe, was highly composed of total phenolic and total flavonoid contents which correlated to the powerful ability of free radical inhibition against DPPH (IC_{50} = 12.98 ± 1.23 µ g/mL), ABTS (IC_{50} = 1.60 ± 0.08 µ g/mL), ferric ion (FRAP value = 5495.43 ± 19.56 µ mol Fe2+/g), as well as delayed lipid oxidation. [8, 27-28] Moreover, it also dose-dependently suppressed MDA levels up to 60% which attributed to the presence of high total phenolic and flavonoid. [8] Apart from these, an antioxidant property has been also found in almost medicinal plants in R-

AMR such as *Aloe vera* [9], *Ferula assa-foetida* [29], as well pulp part of *Tamarindus indica*. [11] Earlier reports have also discussed that its high antioxidant activity may be attributed to several phytochemical constituents such as hydroxyanthraquinones, tannins, gallic acid, and emodin, etc. Our phytochemical screening by LC-MS/MS also indicated different bioactive compounds classified as 19 phenolic and 16 flavonoid compounds including 3-Glucogallic acid, Chebulic acid, Aloin A, Ellagic acid, Apigenin, and so on. These compounds have been greatly investigated as an antioxidant agent [10, 30-31] as well as anticancer property. [32-33] Interestingly, Ampelopsin, which is the bio-compound only found in R-AMR2.2 has been reported to suppress the glioma cell proliferation by modulating G1 and S phase arrest as well as inducing cell apoptosis. [34] Moreover, it previously proved that Ampelopsin has excellent capacity on oxidative stress and inflammation by reducing ROS accumulation, increasing cellular antioxidant defense through activation of the ERK, Akt, and NF-κ B signaling pathways. [35-37] Therefore, the activity of R-AMR on anti-oxidation might result from the ability of these bioactive compounds to scavenge free radicals and protect cells from the serious situation. According to the National Cancer Institute USA, it is mentioned that the cytotoxic effects of natural crude extracts are considered at IC_{50} value ≤30 µg/ml when tested during 48-72 h. [38] Therefore, R-AMR had no cytotoxic effects on HT-29 human colon cancer cells. However, it does not definitely mean that the recipe could not apply for cancer treatment because previous study found a role of antioxidant agents as a chemopreventive agent, the protective compound that can suppress the initiation of carcinogenesis, in reducing oxidative stress and harmful effects in colon cancer. [26] Hence, R-AMR might be a helpful intervention for cancer treatment through an antioxidant mechanism. However, it is essential to further investigate the effect of R-AMR on cell oxidative stress as well as other mechanism effects associated with oxidative stress on cancer cells.

5. CONCLUSIONS

R-AMR is the herbal Thai recipe used for taking care of the overall health status in colon cancer patients clinically. From this current study can be concluded that R-AMR possessed a strong antioxidant activity. Even it has a weak potential activity on killing colon cancer cell lines, however, it may behave as a chemo-preventive agent to prevent cells from ROS-mediated cell damage and also to downgrade the soreness of the disease.

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8. AUTHOR CONTRIBUTION STATEMENT

J.C. designed the research concept proposal, supervised the study, read and approved the final manuscript. T.S. performed all experiments, analyzed the data, and drafted the manuscript. J.Y. designed the experiment of cytotoxicity on cancer cells, analyzed the data, read and approved the final manuscript. T.S. and K.M. provided the plant materials and helped to identify all plant material, read and approved the final manuscript. D.S. helped to analyze the phytochemical data, read and approved the final manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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