ORIGINAL ARTICLE
DOI: 10.4274/tjps.galenos.2023.28092

Phytochemical and toxicological analysis of an herbal mixture containing *Hypericum perforatum* and *Melissa officinalis*

Short Title: Phytochemicals and toxicological analysis of mixure

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10.06.2023
28.08.2023
28.08.2023

Abstract
Objectives: The study aimed to formulate a novel herbal mixture of *Hypericum perforatum* (H) and *Melissa officinalis* (M), and evaluate its toxicity, microbial load, and phytochemical content.
Materials and Methods: Total flavonoids were measured by $AlCl_3/NaNO_2$ complex formation method and colorimetric assay. Quercetin content of the herbal mixture was determined by reverse-phase HPLC. The *in vitro* and *in vivo* safety of the herbal formulation was analyzed using the MTT assay, and acute oral toxicity analysis in the rat model respectively.
Results: The formulated extract (HM), in comparison with rutin used as standard, had a total flavonoid content of 15.29±0.64 mg rutin per ml sample. Reverse-phase HPLC revealed a quercetin content of 0.187 mg/ml. Microbial tests for *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella spp.* were negative. The colony counts for the total aerobic microbial count, and total yeast and mold counts were less than 10 in each case. The MTT assay (with up to about 5 %v/v HM extract) on NIH/3T3 cell line revealed no cell toxicity in the range of concentrations tested. Acute oral toxicity was tested in the Wistar rat model, and the LD_{50} was 695.2 ± 7.5 mg/Kg. HM extract had a dry weight of 38.1 mg/ml.
Conclusion: The preliminary results proved the safety of the herbal mixture HM, with toxicity and microbial load well within the limits of accepted guidelines allowable for use in clinical trials.
Keywords: *Melissa officinalis*, *Hypericum perforatum*, combined hydroethanolic extract, cell toxicity, animal toxicity

INTRODUCTION

In the course of history, man has always looked to nature to provide food, nutrients, and natural substances to treat various illnesses. Traditional medicine was founded based on this relationship between man and nature thousands of years ago.[1] To date, only a small percentage of the total number of plant species on earth has been analyzed phytochemically, and even a smaller percentage has been screened for potential pharmacological use. Despite the recent advances in our fundamental understanding of many disease mechanisms, such as cancer, chronic inflammation, diabetes, and neurodegenerative disorders, there is a great need for development of more effective pharmaceutical solutions. Plant extracts containing many secondary metabolites with various biological/pharmacological activities can affect many targets and can potentially fill this gap. Previous studies have shown that essential oils and extracts isolated from different plant species exhibit powerful antimicrobial and antioxidant, as well as anti-inflammatory, anti-cancer and hepatoprotective activities.[2-6] A significant proportion of all drugs produced worldwide are either plant products or derived from them, and phytopharmaceutical research has played an important part in the discovery and development of many more synthetic drugs.[7] At the same time, mixed herbal extracts can be used as multi-target drugs that act synergistically, have fewer

side effects, and in the process lower the costs of treatment.[8]

Lemon balm, with the scientific name *Melissa officinalis* (M), is a well-known perennial herbaceous plant belonging to the family Lamiaceae which has been studied extensively for its medicinal powers. It is native to Asia, North America, the Mediterranean, and Southern Europe, and is extensively cultivated in these regions.[9] The leaves of M have historically been used to treat a number of ailments including digestive and inflammatory diseases, and microbial infections.[10] The presence of phenolics, terpenoids and flavonoids such as quercetin, rutin and quercetrin in the extract of M are thought to be responsible for its ability to treat various diseases.[11] The essential oil contains important secondary metabolites such as geranial, cineol, and caffeic acid, capable of reducing serum cholesterol and triacylglycerides. Flavonoids are known for their powerful antioxidant activities and are also present in M extract.[12] *Hypericum perforatum,* or St. John's wort (H), is another medicinal plant of great importance that has been used for thousands of years. This perennial herb belongs to the Hypericacea family and is native to temperate regions of the world. It has been studied extensively due to its importance as a powerful medicinal plant that can treat various illnesses.[13] Leaves of the plant are known to contain active metabolites such as flavonoids, naphthodianthrones and phloroglucinols. Antidepressant effects of H extract are comparable to common synthetic antidepressants in mild to moderate cases.[14] Many studies have focused on the antiviral activities of H and the results seem to be promising.[15,16]

Each of these plants has a multitude of secondary metabolites that can exert a positive influence on treating various diseased states. A question that comes to mind is whether a combined extract of these two plants formulated for clinical testing could be more effective and maximize their therapeutic efficacy. Previous studies have confirmed that combining herbal formulations is generally associated with an increase in the functional properties and biological activities of the mixed extracts.[17,18] However, scientific data on the phytochemical analysis of the combined extract of M and H, and in particular determination of cell and animal toxicities, is scant. In the current study, a new formulation of the combined ethanolic herbal extract of H and M was prepared in order to do the preliminary safety and phytochemical analyses in preparation for future clinical testing.

MATERIALS AND METHODS

Plant Acquisition and Extraction

Both H and M herbal samples were obtained from a medicinal plant farm in Ardabil, Iran. They were carefully cleaned of debris and air-dried in the laboratory. Herbarium samples of the plants were sent to the Institute of Pharmacology, XXX University of Medical Sciences for identification. After proper identification, samples were registered and code numbers were assigned (PMP-2310 and 14001 for H and M, respectively). After weighing, plant samples were ground together in a pharmaceutical grinding mill to an adequate size for extraction (coarse ground). An Accelerated Solvent Extraction system (ASE) was used to perform the extraction with 70% pharmaceutical-grade ethanol in water. Preliminary test trials indicated no need for elevated extraction temperatures and a pressure of 10MPa was used to perform the extraction. The mixed ground plant tissue was loaded into extraction vessels and was allowed to fill with extraction solvent. The system was allowed to sit for static extraction for 24 hours, after which dynamic extraction was resumed to completion. The extraction solvent was fluxed through the ASE system two more times (i.e., a total of three times). The final product (HM Extract) was used for subsequent analysis in the study. To determine the dry weight, 10 ml of HM extract was dried in triplicates at 35-40ºC in a drying oven until no further weight change was noted. The total volume of extraction, and the final percent of ethanol present in the extract, was also measured and recorded.

Phytochemical Analysis

Total Flavonoid Content

Aluminum chloride/sodium nitrite method was used to determine the total flavonoid content of the HM extract.[19] In this method o-nitroso derivatives of flavonoids form a complex with Al III which absorbs maximally at or near 510 nm. Rutin (Sigma-Aldrich, USA) was used as the standard to determine the total flavonoid content of HM as µg rutin/ml. Sodium hydroxide (4%), sodium nitrite (5%), and 10% aluminum chloride in deionized water were used to do the procedure. Samples were prepared by adding 25 µl (1:10 dilution) of HM extract to 100 µl water and 7.5 µl sodium nitrite solution, into a 96-well ELISA plate in 8 replicates. After six minutes, 7.5 µl aluminum chloride, 100 µl sodium hydroxide, and 110 µl of deionized water were added to each well and covered with aluminum foil for 15 minutes. Absorption at 510 nm was then read and recorded in ELISA reader (Synergy, Biotek Instruments Inc., Germany). The same procedure was repeated for different concentrations of rutin standard. The 6 best results were selected for total flavonoid determination.

Determination of Quercetin concentration by reversed-phase high-performance liquid chromatography (RP-HPLC)

Quercetin, a key marker and constituent of both H and M, was analyzed with HPLC. RP-HPLC is routinely used for optimal separation of flavonoids due to their hydrophobicity and low solubility in

aqueous solutions.[20] All HPLC grade solutions and equipment were obtained from Alborz Academic Institute, Iran. To prepare the standard solution, 10 mg of quercetin dihydrate (Sigma-Aldrich, USA) was dissolved in 20 ml methanol, 15 ml dilute hydrochloric acid, 5 ml water and the final volume was adjusted to 50 ml with methanol. To make the sample solution, 15 ml HM extract was first dried. The dry extract was then added to the same series of solutions as in the standard with a final volume of 50 ml as described above. A 25 cm C18 column (Phenomenex, USA) with a 4 mm diameter and 5 µm particle size was used to do the HPLC on an Alliance E2695 (Waters, USA). Gradient elution was set up with a mobile phase consisting of 0.3 g/L phosphoric acid (solution A) and pure methanol (solution B). The gradient started with 60% solution A, and 40% solution B, and ended with 0% A and 100% B. The injection volume was 10 µl and the running time was 25 minutes at 25ºC. The detector was set at a wavelength of 370 nm with a flow rate of 1 ml/min. Quercetin identification was done by comparing retention times of sample peaks with the quercetin peak in the standard.

Cell Culture and Cell Toxicity

MTT (3-(4,5-dimethyl thiazolyl)-2,5-diphenyl tetrazolium bromide) assay was used to assess the cell toxicity of HM hydroethanolic extract in NIH/3T3 cell line based on general protocols described in ISO-10993-5 and Dnihelova and colleagues.[21] The NIH/3T3 cell line was obtained from The Iranian National Center for Genetic and Biologic Resources. The cell culture medium was complete DMEM with 10% FBS, 10 IU/ml penicillin and 100 IU/ml streptomycin (Capricorn, Germany). Standard growing conditions consisted of 5% CO2 and 95% humidity at 37ºC. After three passages, cells were grown in T175 cell culture flasks to confluence. They were then trypsinized and centrifuged at 1200 rpm for five minutes. After resuspending the cells in the cell culture medium, viable cells were counted on a hemocytometer aided by trypan blue to identify non-viable cells. An average number of 2×10^4 cells were incubated (24 hours at 37ºC) in wells of a flat-bottom ELISA plate, and used to do the MTT assay. The culture medium was replaced with various concentrations of HM extract (0.5 to 50% v/v) which was added to NIH/3T3 cells and incubated at 37ºC for 24 hours. The control wells received media containing complete DMEM with FBS and antibiotics, but no HM extract. To do the MTT assay, the cultured medium was removed from each well, and cells washed with 100 µl of fresh DMEM. To each well, 50 µl of cell culture medium and 50 µl 5 mg/ml MTT reagent (Sigma-Aldrich, USA) was added, and the flasks were incubated for 4 hours at 37ºC. MTT is a yellow solution converted to formazan crystals by mitochondrial NADPH-dependent oxidoreductases in metabolically active cells. To dissolve the formazan crystals, 150 µl DMSO was added to each well for 15 minutes on a shaker. A blank containing MTT reagent and DMSO was used to adjust the baseline absorption. Absorbance at 570 nm was read and recorded in an ELISA reader (Synergy, Biotek Instruments Inc., Germany). Results were expressed as % control ± SEM.

Acute Oral toxicity, LD_{50} in Wistar Rats

Acute oral toxicity of HM was studied in Wistar rats obtained from the Institute of Pharmacological Sciences, XXX University of Medical Sciences (Iran). Procedural and ethical considerations were based on OECD UDP Procedure 425 (2020). Animal procedures were approved by PNU Ethics Committee. Animals were housed under standard conditions of 12/12 hour light/dark cycle without food or water restrictions at 25ºC. All animals were acclimated to standard conditions for ten days prior to dosing. The average weight of the animals was 200-250 grams, and all doses were calculated according to kilograms body weight. Animals were singly dosed in the test stage using default doses recommended by OECD guidelines (50, 100, 500, 1000 mg/Kg) to find a suitable range of dose/response for the main stage of the experiment. After initial dosing, animals were monitored for 72 hours for signs of toxicity. The HM doses selected for the main stage of the experiment were 0, 381.0, 571.5, 685.8, and 762.0 mg/Kg administered by gavage. The main stage consisted of 1 control and 4 treatment groups, with 10 rats in each group (n=10). Clinical manifestations of toxicity as well as mortality, were monitored and recorded for 14 days. LD_{50} was calculated with SPSS 26 software/Probit analysis.

Analyses of Microbial Content

In order to analyze the microbial load of HM extract, the USP-40 general protocol for analysis of non-sterile products was used. Serial dilutions of 1:10, 1:100, and 1:1000 of HM extract was prepared in peptone water buffer, and the total aerobic microbial count was done by adding 1 ml of each dilution to 15 ml plate count agar in 9 cm Petri dishes in duplicates. The incubation duration was 5 days at 30ºC. The same procedure was used to do a total yeast and mold count on casein-soybean digest agar. Presence of the four main groups of food pathogens, i.e., *Escherichia coli, Salmonella spp., Staphylococcus aureus,* and *Pseudomonas aeroginosa* in HM extract was explored on MacConkey, bismuth sulfite, mannitol salt and cetrimide agars respectively. Uninoculated negative control plates were run in parallel, and results were recorded as a number of colony-forming units per ml (cfu/ml).

Statistical Analysis

One-way analysis of variance (ANOVA) and Probit analysis of LD_{50} were done using SPSS 26 software.

Dunnett test was done as a post hoc analysis method where $P<0.05$ was statistically significant. The final results were shown as mean ± SEM.

RESULTS AND DISCUSSION

Phytochemical analysis

The data for total extraction volume, percent ethanol, and dry mass of HM extract is presented in Table 1. Total flavonoid content was determined using the aluminum chloride/sodium nitrite method by measuring A_{510}. Comparing the A_{510} of HM sample with the rutin standard curve revealed a total flavonoid content of 15.29±0.64 mg rutin/ml HM extract ($^{*}P<0.05$). HM extract contains a significant amount of flavonoid (40.1% of total dry weight) compared to levels found in each plant individually according to related studies.[22,23] Many factors such as habitat and environmental conditions during plant growth, sample preparation, extraction methods, and analytical techniques, can affect secondary metabolite concentrations in plant extractions. Quercetin levels in HM (0.187 mg/ml) were in agreement with results obtained in similar studies.[20,24]

Eighteen peaks were detected in the reverse-phase HPLC chromatogram of HM. The quercetin peak in the HM sample (peak number 18, chromatograph b) was identified by comparing peak retention times with the peak for quercetin standard (chromatograph a). Areas under the peaks were compared, and quercetin concentration per ml of HM extract was calculated relative to the standard. These numbers were corrected for the dilutions to determine the quercetin concentration in the HM sample. The quercetin concentration calculated was 0.187 mg/ml.

Microbial content assays

The presence of four main pathogenic contaminants of food and non-sterile products, i.e., *E. coli*, *Pseudomonas aeruginosa*, *Salmonella spp.*, and *Staphylococcus aureus,* was tested, with negative for growth in all cases. Total aerobic microbial count (TAMC) and total yeast and mold count (TYMC) yielded negative results. The data for microbial load tests was summarized in Table 4. According to USP-40 guidelines for microbial testing of non-sterile pharmaceutical products, HM extract passes the requirements for human consumption.

Toxicity assays

Cellular Toxicity: The MTT assay was used to determine cellular toxicity of HM in NIH/3T3 cell line. Results are summarized in Table 5 and Figure 2. Cellular toxicity was measured by colorimetric assay of formazan crystals produced by viable cells. No toxicity was noted in the range of concentrations tested. However, there was a significant concentration-dependent increase in apparent cell viability above 5% v/v of HM, which may be an indication of interference with the assay. Such effects are commonly seen with electron transport chain uncouplers or molecules that can directly reduce the MTT reagent to make formazan crystals independent of mitochondrial oxidoreductases. Examples include NADH, ascorbic acid, glutathione, and flavonoids which are powerful antioxidants present in significant amounts in HM extract. This would explain the dose-dependent increase in absorption level observed at HM concentrations higher than %5 v/v.

Animal toxicity: The results for acute oral toxicity (LD_{50}) in Wistar rats are summarized in Table 6. Animals were monitored for 14 days for visual clinical manifestations of toxicity (Table 7) following the administration of HM extract by gavage. The data indicates an LD_{50} of 685.8 mg/Kg. The SPSS software was used to do a non-linear regression analysis (PROBIT analysis) of the LD_{50}. The software calculated an LD_{50} of 695.2 ± 7.5* mg/Kg ($p<0.05$) with a 95% confidence interval of 599.4-913.3 mg/Kg. The only clinical signs noted were abdominal distension and diarrhea in the dose range of 571.5-685.8 mg/Kg, as shown in Table 6. The maximum dose without any signs of toxicity was, therefore, 381.0 mg/Kg of HM extract.

These results are statistically significant. Various animal studies on Lemon balm have reported an LD_{50} in the range of 2000-3000 mg/Kg.[25-27] Other studies on St. John's wort toxicity in the animal model report an LD_{50} in the 1000-3200 mg/Kg range.[28-30] Therefore, HM extracts with an LD_{50} of 695.2 ± 7.5 mg/Kg are more toxic than the individual extracts of each plant. This toxicity level places the HM extract in category 4 (medium toxicity, between 500-2000 mg/Kg), which is less toxic than caffeine, aspirin, or ibuprofen. In accordance with the concentration and diversity of secondary metabolites present in HM extract, it is expected that it should also have different pharmacological activities than the extract of each plant. No significant weight change or changes in the feeding pattern of the animals were noted during the 14-day duration of the experiment. The only visible clinical parameters noted were abdominal distension and diarrhea in the HM dose range of 571.5-685.8 mg/Kg. Lemon balm and St. John's wort have been shown to be antispasmodic and antidiarrheal and are used to alleviate abdominal discomfort.[31,32] Therefore, it is possible that at higher doses of HM, abdominal distension and diarrhea are absent due to these effects.

CONCLUSION

The diverse array of secondary metabolites found in plant extracts seems to have unlimited potential

and possibilities. In the last few decades utilization of plant extracts and products to treat various diseases has been successful in many cases, and results in this area seem promising. Therapeutic strategies using phytopharmaceuticals provide a new perspective to fill the void that chemical drug design and manufacturing worldwide has not been able to fill. HM is a new formulation of the combined hydroethanolic extracts of Lemon balm and St. John's wort, which was analyzed for its phytochemical properties, and cell and animal toxicities in the present study. HM contains flavonoid in high concentrations. No microbial growth was observed in microbiological tests performed on HM. At concentrations of up to 1% v/v, HM did not result in toxicity in NIH/3T3 cell line and it did not interfere with the MTT assay. Higher concentrations of HM seem to have interfered with the MTT assay and resulted in apparent cell viability greater than the control. PROBIT analysis of the acute oral toxicity of HM extract in Wistar rats indicated an LD_{50} of 695.2 mg/Kg body weight which places HM in toxicity category 4 (moderate toxicity). Taking all the results into consideration, a range of 1-5% of the maximum dose of HM without any indications of toxicity in the animal model (i.e., 3.81-19.1 mg/Kg/day) is suggested for pharmacological testing in clinical trials.

ACKNOWLEDGEMENTS

The authors express their deepest gratitude to professor XXX at XXX University of Medical Sciences, and XXX Pharmaceutical Company, Tehran, for their guidance and technical support.

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Table captions:

Table 1: Percent alcohol, volume, and mass of dried extract per ml HM extract

Total volume extracted	% Alcohol	Dried Extract
1320 ml	58%	38.1 mg/ml

Table 2: Absorbance at 510 nm of different concentrations of rutin used to construct the standard curve.

Rutin Standard (µg/ml)	A510 ± SEM
0.0	0.000 ± 0.001
14.2	0.044 ± 0.001
28.6	0.082 ± 0.001
35.7	0.100 ± 0.001
71.4	0.171 ± 0.001
142.9	0.333 ± 0.001
178.6	0.410 ± 0.001

Table 3: Total flavonoid expressed as mg standard rutin (*P<0.05)

Total flavonoids in HM, corrected for 1:140 dilution SEM ± mg/ml	Total flavonoids as mg standard rutin SEM ± µg/ml	A510 ± SEM
15.29 ± 0.64*	109.2 ± 4.6*	0.259 ± 0.001

Table 4: Summarized results of microbiological tests

Test	Results	Acceptable range	Standard	unit
Pseudomonas aeroginosa	-	-	USP 40	cfu/ml
Staphylococcus aureus	-	-	USP 40	cfu/ml
Salmonella spp.	-	-	USP 40	cfu/ml
E. coli	-	-	USP 40	cfu/ml
Total Yeast and Mold (TYMC)	)*(10<	Max 10^2	USP 40	cfu/ml
Total Aerobic Microbial count (TAMC)	)*(10<	Max 10^3	USP 40	cfu/ml

Significant differences were assessed by *P < 0.05.

Table 5: MTT assay results for cell toxicity in NIH/3T3 cell line

Concentration %v/v	**0**	**0.5**	**1**	**5**	**10**	**25**	**50**
Mean (% control) ±SEM	99.20±2.11	103.55±3.17	119.52±3.31	237.10±3.67*	248.07±5.65*	395.00±2.69*	594.20±18.4*

Significant differences were assessed by *P < 0.05. Results are expressed as % control.

Table 6: Raw data for acute oral toxicity (mortality for 14 days) with single dose of HM extract in Wistar rats

Dose (mg/Kg)	0	381.0	571.5	685.8	762.0
Mortality	0	0	3	5	6
Mortality (%)	0	0	30	50	60

Table 7: Visible clinical manifestations of toxicity with HM treatment in Wistar rats

Visible clinical manifestations	0 mg/Kg (control)	381.0 mg/Kg	571.5 mg/Kg	685.8 mg/Kg	762.0 mg/Kg
Skin	-	-	-	-	-
Eyes	-	-	-	-	-
Abdominal distention	-	-	+	-	-
Diarrhea	-	-	+	+	-
Respiratory	-	-	-	-	-
Arrhythmias	-	-	-	-	-
Hair	-	-	-	-	-
Mobility	-	-	-	-	-
Paralysis	-	-	-	-	-
Pain	-	-	-	-	-

Animals were monitored for 14 days and results were recorded.

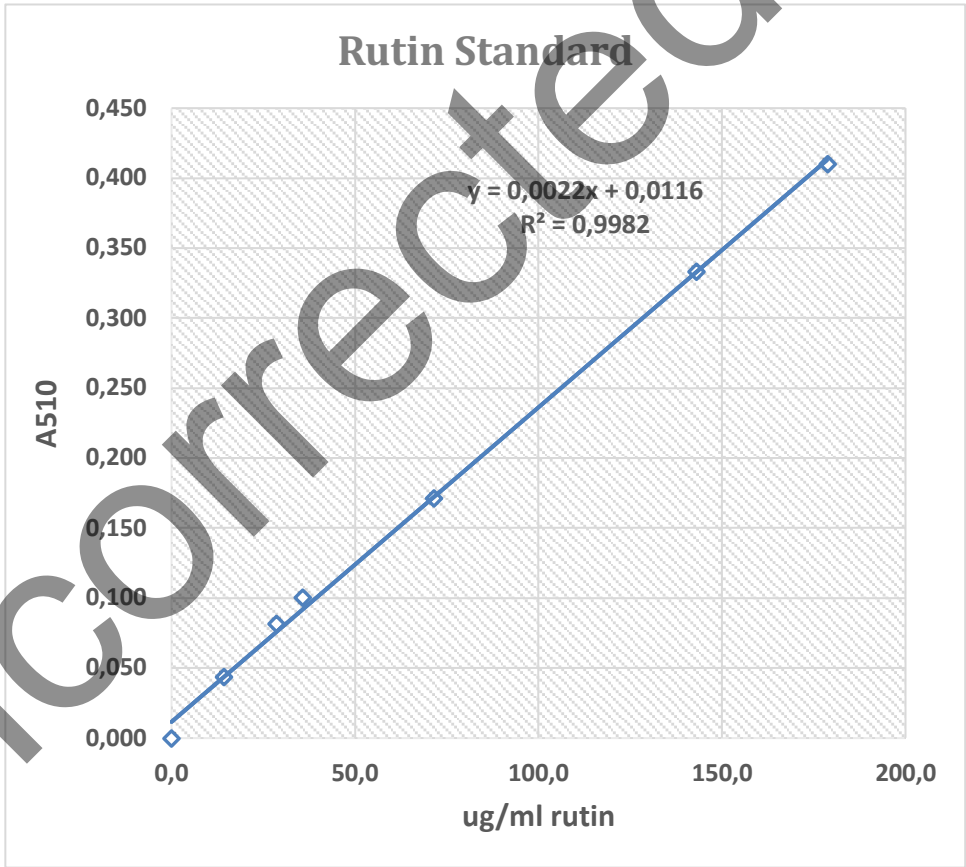

Figure 1: Rutin standard curve. The total flavonoid content of HM was determined by linear regression analysis

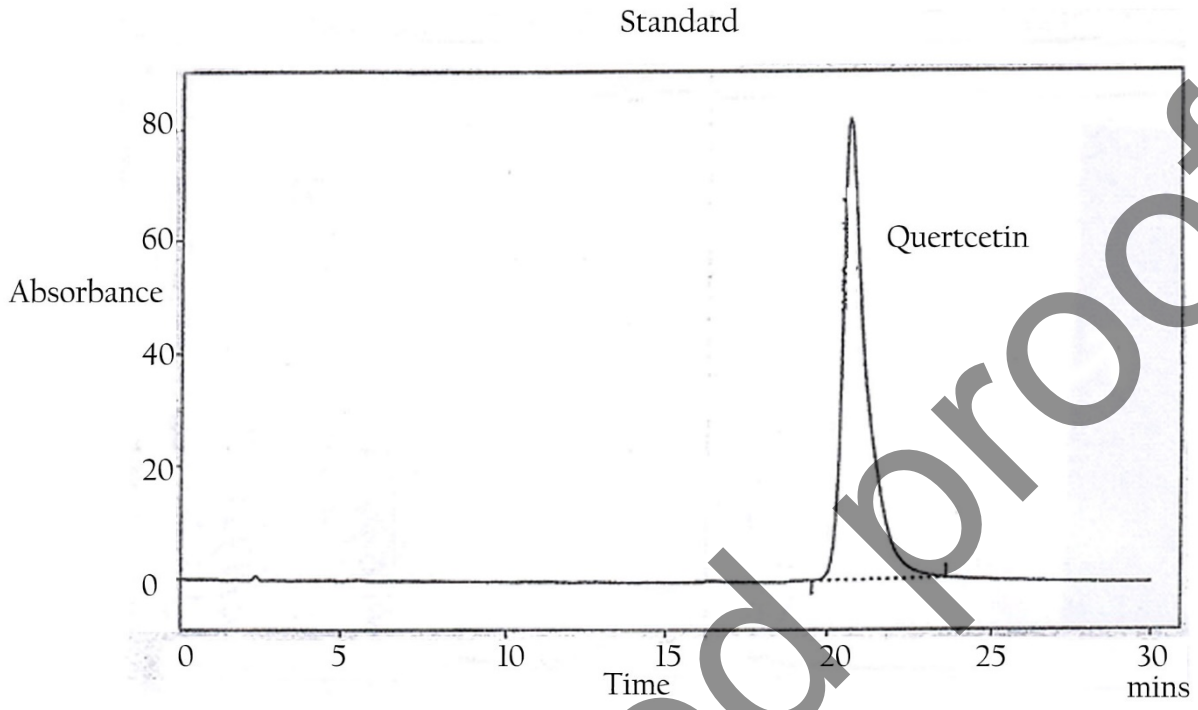

Figure 2a: Chromatograph of standard quercetin, concentration 200mg/ml

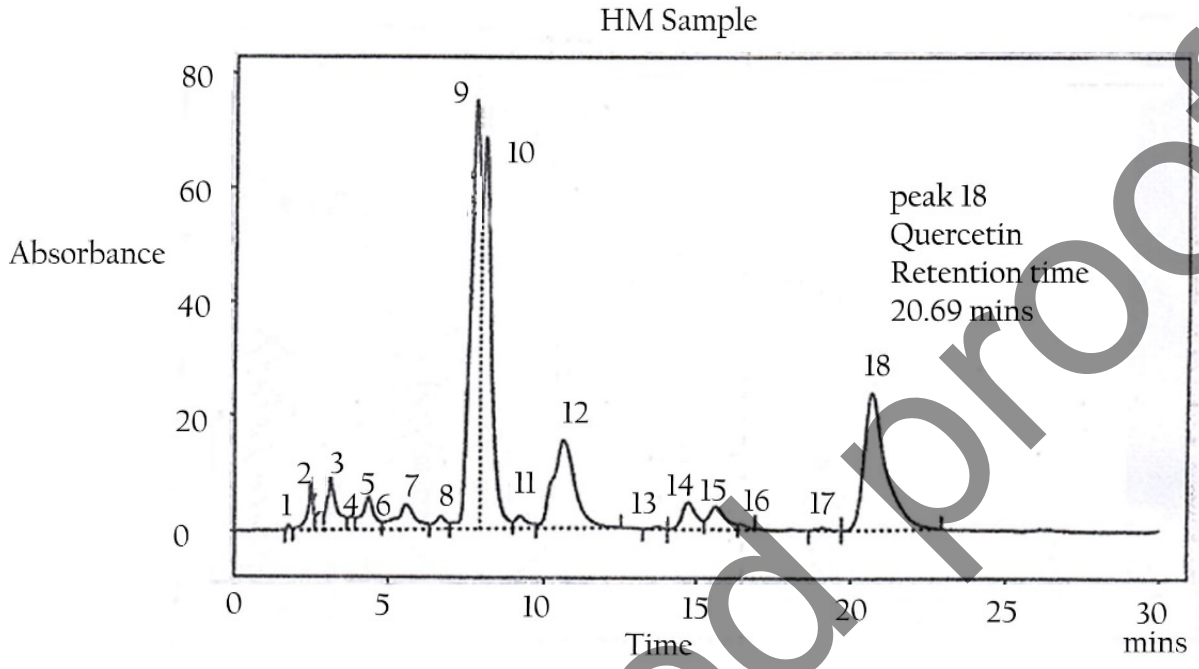

Figure 2b: Chromatograph of HM sample. The Quercetin peak is indicated with an arrow

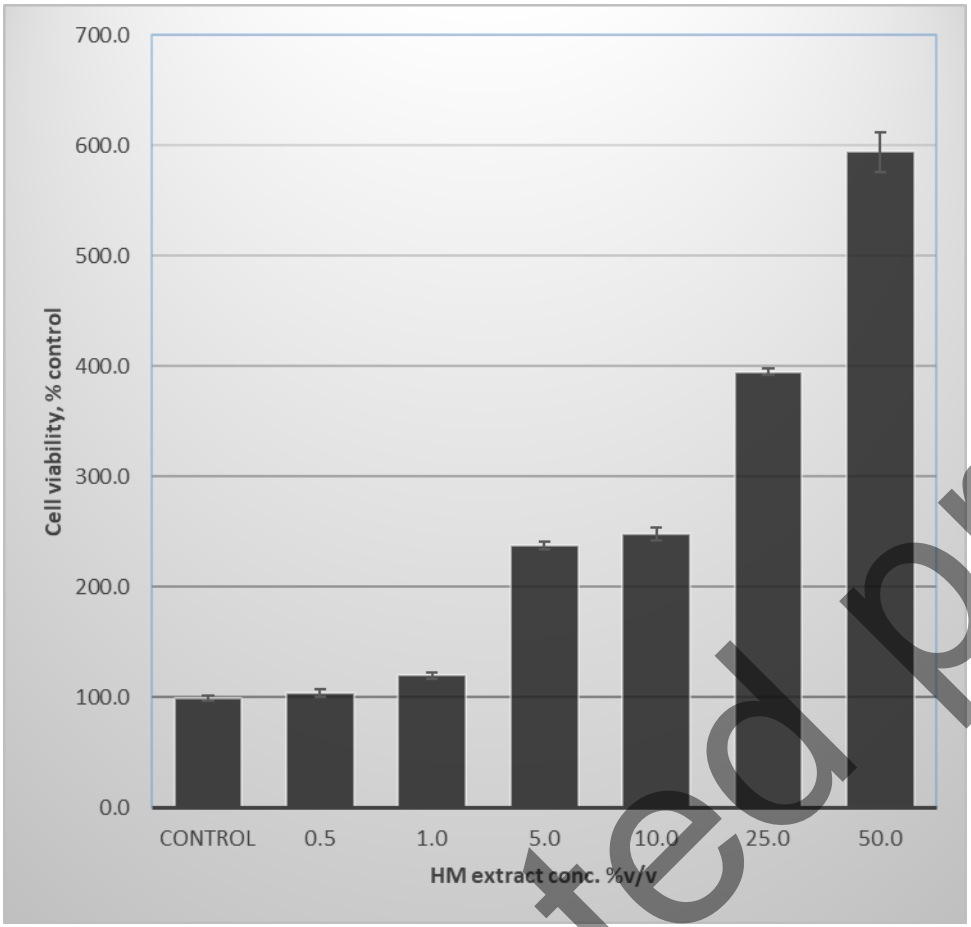

Figure 3: Graphical results of the MTT viability test of NIH/3T3 cell line in response to various concentrations of HM extract, expressed as % control.