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Research Paper

In vitro Cytotoxicity and Antioxidant Activity of Butanolic Extract from Root of *Marrubium vulgare* L. on Human Cancer Cell Lines

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ABSTRACT

Root of *Marrubium vulgare* was evaluated for quantitative estimation of phytochemicals (Phenol, flavonoid and terpenoid) and antioxidant activity using spectrophotometric methods. Methanolic root extract and its Butanol fraction were also assayed for its cytotoxicity against three cancer cell lines. The obtained results showed that butanol fraction (But fr) contains highest amount of both terpenoids and phenols (667.36±23.69 mg linalool/g DW and 402.356± 2.89 mg GAE/g DW). In addition, both MeOH extract and its BuOH fraction showed strong antioxidant activity against DPPH (IC_{50} = 54.84±3.05 and 33.93± 0.93 µg/mL) as well as significant cytotoxic effect with an IC_{50} value ranging from 1.414 to 6.286 µg/mL against all the three-cancer cell line (A549, MCF-7 and PC-3). These results reveal that methanol extract of root and its butanolic fraction constitutes an important source of antioxidant and can be evaluated for potential promising anticancer activity.

***Key words*:** Total phenol, Cytotoxicity, *Marrubium vulgare*, Butanolic extract, DPPH

Genus Marrubium with promising chemical and biological properties of which *Marrubium vulgare* L. (White horehound) an important species has been studied the most amongst forty species; the reason being its wide usage in folk medicine. This perennial herb distributed in Europe, Asia and Mediterranean region is mostly used to cure a variety of diseases like asthma, liver problems, cholera and prolonged fevers [1-4]. Leaves have been used in inflammation, sore eyes, night blindness, strengthen the teeth, and facilitate the expulsion of foetus [5]. *M. vulgare* is also used for flavoring beverages and candies in USA [6-7]. Syrup containing leaves and stems has been used to cure chronic coughs in asthmatic or short-winded patients. An infusion of leaves is given as an insecticide and against caterpillars [8].

Pharmacological activities including anticancer [9] antispasmodic [10], antidiabetic, [11] hepatoprotective [12] gastroprotective [13] and antimicrobial [14], so far has being reported and considered due to presence of good amount of diterpenes, sesquiterpenes, flavonoids and phenylpropanoid esters. In the present study we screened the root extract and its fractions for cytotoxicity against different human carcinoma as well as normal (FR2) cell line so an anticancer drug could be formulated.

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MATERIALS AND METHODS

The whole plant *M. vulagre* L. was collected from Malkha nowhatta (Srinagar) in the month of May 2018. The identification of the plant was done on the basis of characteristics described by [15] and authenticated by Dr. Anzar Ahmad Kharoo (Centre for Biodiversity & Taxonomy, Department of Botany, University of Kashmir). A sample of the plant material was deposited in the herbarium of the Department of Taxonomy, University of Kashmir under voucher specimen number, *Marrubium vulgare*- 2678 KASH [Ref No: F (voucher-specimen CBT/KU/18)] for future reference. From the collected plant material, each part (root, stem, leaves and floral part) was separately shade dried in a dark, well ventilated room for four weeks. The dried plant material was coarsely pulverized to powdered by grinder, only extraction of root was done, the rest samples were stored till further use.

Preparation of plant extract

Air dried root (100 g) was subjected to maceration using methanol (1 L) as solvent and the process took at least 4-5 days. The extract was filtered through Whatman filter paper No.1 then concentrated under vacuum at 40°C using rotary evaporator to get 6.5 g methanol extract. Later this

methanol extract (6.5 g) was dissolved in water and sequentially fractionated into n-hexane, butanol, chloroform and aqueous to obtain their respective fractions. The solvent of each was evaporated under vacuum to dryness and stored at 4°C until analyzes.

Quantitative estimation of phytochemicals

Determination of total terpenoid content

Total terpenoid content was determined through colorimetry using the procedure followed by [16] with some modifications. Briefly, 1.5 ml chloroform was added to 200 µl of MeOH extract and its hexane, butanol, chloroform and aqueous fractions, well mixed then allowed to rest for 5 min. Concentrated H_2SO_4 (100 µL) was then added to each sample taken in 2.5 mL appendorf, incubated in dark for 2 h at room temperature. After 2 hours of incubation, dark reddish-brown precipitate settled down indicated the presence of terpenoids. The supernatant was carefully decanted and the precipitate was dissolved in 1.5 ml methanol. 100 µl from each sample in appendrof was transferred in a 96 well plate for spectrophotometric analyses. Linalool (1.56 – 100 mg, R^2 = 0.999) was used to prepare a standard curve. The Absorbance was recorded at 538 nm in a spectrophotometer against a methanol. The assay was performed in triplicate and concentration was expressed as equivalent to mg linalool/ g of DW.

Determination of total phenolic content

Total phenolic content was assessed by the Folin-Ciocalteu spectrophotometric method [17] in a 96 well plate with slight modifications. In brief 50 µL of the diluted methanolic extract and its hexane, butanol, chloroform and aqueous fractions (5 mg/ mL) were mixed with 100 µl of 1:4 diluted Folin–Ciocalteu reagent and shaken for 1 minute in a flat-bottom 96-well microplate. The mixture was left for 5 minutes and then 75 µL of 20% sodium carbonate solution was added and the mixture was shaken at medium continuous speed for 1 min. After 2 h incubation at room temperature, the absorbance of the reaction mixture was measured at 765 nm using the microplate reader Tecan Infinite M Nano Elisa plate Reader (Austria). Serially diluted Gallic acid (0.78, 1.562, 3.125, 6.25, 12.5, 25, 50, 100 µg/mL, R^2= 0.991) was used as standard for calibration. Total phenolic contents were expressed as mg Gallic Acid Equivalents (GAE) per g DW.

Determination of total flavonoid content

Total flavonoid content was determined by aluminum chloride colorimetric method adopted by [17] with slight modification. 50 uL of extract solution (5 mg/mL in methanol) was added to 10 µl of 10% aluminum chloride solution and followed by 150 µL of 96% ethanol. 10 µl of 1 M sodium acetate was added to the mixture in a 96 well plate. 96% ethanol was used as blank. Standard solution of Quercetin (0.78, 1.562, 3.125, 6.25, 12.5, 25, 50, 100 µg/mL, R^2= 0.989) in methanol was also prepared with the same procedure. Total flavonoid contents were expressed as mg Quercetin equivalent/g DW.

Antioxidant activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay of the crude methanolic extract and its fractions (Hx fr, But fr, Chf fr and Aq fr of root) were determined using the method described by [18]. In a 96 well plate a volume of 100 mL of 5 samples (M ex, Hx fr, But fr, Ch fr and Aq) at various concentration (1000- 15.62 ug/ml) were added to 100 µL of a methanol solution of DPPH (0.1 mM) in each well. The reaction mixture was incubated for 30 min.at room temperature in dark and the absorbance was measured at 517 nm using micro-plate reader of Tecan Infinite M Nano Elisa plate Reader (Austria). Ascorbic acid Ascorbic acid was used as positive standard and methanol as positive control. All tests were performed in triplicates and radical scavenging ability (%) was calculated as follows:

$$\% \text{ Scavenging} = [(A_{\text{positive control}} - A_{\text{sample}})/(A_{\text{positive control}})] \times 100$$

where $A_{\text{positive control}}$ is the absorbance of the control reaction (containing all reagents except the test extract or standard), and A_{sample} is the absorbance of the test extract or standard.
Concentration of samples (extracts and standard) resulting in 50% inhibition on
DPPH (IC_{50} value) were calculated using GraphPAD Prism Software Version 5.0.

Statistical analysis

Variation in concentration of total phenol, flavonoid and terpenoid content among different plant parts and their antioxidant activities were analyses by both one way and two-way ANNOVA at 5% level. These analyses were performed in Graph pad prism 5.0.

Cell culture and growth conditions

Human lung cancer cell line (A549), breast (MCF-7), prostrate (PC-3) melanoma were obtained from NCI: National Cancer Institute, USA. A-549, MCF-7 and PC-3 cell lines were cultured in RPMI-1640 medium. Culturing of the cancer cells were done in RPMI-1640 medium containing 10% foetal calf serum (FCS), penicillin (100 mg/mL) and streptomycin (100 µg/mL). Standard culture conditions were employed. The cell cultures were grown in CO_2 incubator (New Brunswick, Galaxy 170 R, Eppendorf)) at 37°C with 98% humidity and 5% CO_2 gas environment.

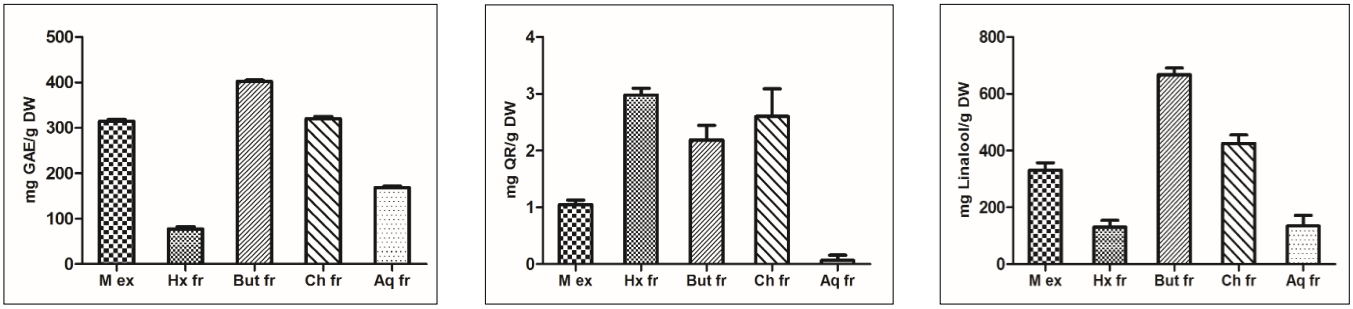
Cytotoxicity assay

The SRB (Sulforhodamine B) [19] assay was used in this study to assess growth inhibition. This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. For the assay a final cell suspension of optimum cell density (7000-12,000 cells/100 µL) was seeded in 96 well flat bottom plates for A549, MCF-7 and PC-3 cell lines (100 µL of cell suspension) and incubated for 24 h. After 24 h of incubation under culture conditions, the cells containing complete growth medium along with known cytotoxic agent paclitaxel, as positive controls were treated with serial concentrations of MeOH extract of root and its butanol fraction (25-0.781 µg/mL). The plates were again incubated under the same conditions for another 48 h at 37°C. Further, cells were fixed with ice cold TCA (trichloroacetic acid) for 1 h at 40°C. After 1 h, the plates were rinsed five times with tap water and allowed to air dry. After drying, 100 µL of 0.4% SRB dye was added for 30 min at room temperature. Plates were then washed 5 times with 1% v/v acetic acid to remove the unbound SRB. After drying at room temperature, the bound dye was solubilized by adding 100 µL of 10 µM TRIS (tris(hydroxymethyl)aminomethane) buffer (pH-10.4) to each well. The plates were kept on the shaker for 5 min to solubilize the protein bound dye. Finally, OD was taken at 540 nm in a microplate reader (Thermo Scientific). IC_{50} was determined by using GraphPAD Prism Software Version 5.0.

RESULTS AND DISCUSSION

Total phenol, total flavonoid and total terpenoid content

Total phenol, flavonoid and terpenoid contents presented in (Fig 1a-c), respectively showed that butanolic fraction contains highest concentration of both terpenoids and phenols (402.36 ± 2.89 mg GAE/g extract and 667.36 ± 23.70 mg linolool/g extract); however, flavoinod content was found highest in Hx fraction (2.98 ± 0.12 mg QR/g extract)



(**a**) (**b**) (**c**)

Fig 1 Total phenol (**a**) total flavonoid (**b**) and total terpenoid (**c**) content in the root extract and its fractions
All values are presented as mean ±SD and the means are significantly different (p<0.001) as determined by one way ANOVA

Antioxidant activity

DPPH radical scavenging activity of *M. vulgae* root extract and its fractions being represented in (Fig 2) showed highest % of inhibition capacity exhibited by butanol fraction (89.92%). All the samples showed concentration dependent increase in radical scavenging capacity with highest IC_{50} inhibition was recorded in butanol fraction (33.93 ± 0.93). The IC_{50} values of methanol extract and its fraction compared with standard ascorbic acid is presented in the (Table 1) given below:

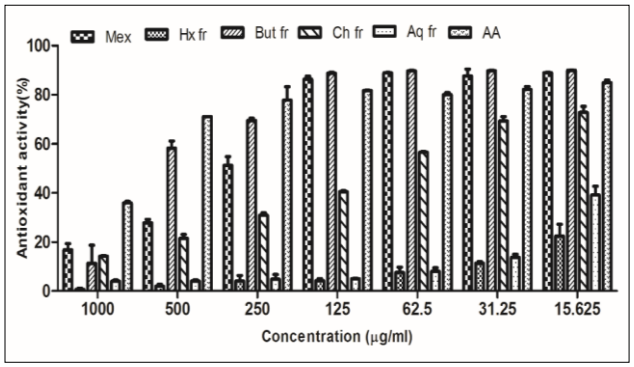


Fig 2 DPPH radical-scavenging activity of the root extract and its fractions. Data was analyzed using a two-way ANOVA (analysis of variance). Significant difference (p<0.05) was observed

Table 1 Inhibitory concentration 50 (IC_{50} in mg/mL) of *M. vulgare* root samples and standard

Sample	IC_{50} (µg/mL)
Methanol extract	54.84 ± 3.05
Hexane fraction	>1000
Butanol fraction	33.93 ± 0.93
Chloroform fraction	193.2 ± 5.4
Aqueous fraction	>1000
Ascorbic acid[a]	15.85± 1.42

[a]Ascorbic acid was used as standard

Cytotoxicity

The result depicted in table 2 summarizes the cytotoxic effect of MeOH extract of root and its Butanolic fraction on A549, MCF-7 and PC-3 cell lines. Among these, MeOH extract was the most cytotoxic against PC-3 cell line with an IC_{50} value of 1.414 µg/mL, and Butanol fraction against MCF-7 (2.494 µg/mL). Moreover, important thing is that both extract and its fraction was found less cytotoxic with an IC_{50} value of above 25 µg/mL.

Table 2 Cytotoxicity data of Methanol extract of root and its Butanol fraction against three cancer cell lines

Sample	A 549	MCF-7	PC-3	FR2
MeOH extract	5.974	1.654	1.414	>25
BuOH fraction	6.286	2.494	3.144	>25
Paclitaxel[b]	<0.01	0.049	0.065	0.005

[a]Results are expressed as IC_{50} in µg for MeOH ex and BuOH fr
[b]Paclitaxel was used as positive control

Antioxidant capacity, a widely used parameter by researchers to correlate the free radical scavenging ability and biological activities is based on the ability of natural products to donate free electrons is measured by DPPH assay. Earlier report submitted by [20] have shown 50% inhibitory concentration value of leaf extract of different locations ranging from 35.7 to 774 µg/mL as against standard ascorbic 63.9 µg/mL; however, in our present study an IC_{50} value of 33.93 µg/mL was observed by butanolic fraction of root. Quantitative estimation of phytochemiclas (phenol, flavonoid and terpenoid) indicates that the proportion of phenolics is much higher than flavonoids. In previous studies [21-22] lower content of phenolics but higher flavonoids were observed compared to the present study. So far cytotoxicity of root extracts obtained from root of *M. vulgare* has not been reported till date, though related study has been done but of the aerial parts of the herb [23-24]. In these studies, cytotoxic effect of alcoholic extract and Acacetine, apigenin and acacetine-7-rhamnoside were assessed against various carcinoma cell lines including MCF-7 and PC-3. The main goal of our study was to develop an anticancer drug, a relationship between antioxidant potentiality and anticancer activity and to identify the bioactive compound responsible for the activity. In the present study as it is evident that Butanolic fraction showed significant results both against cancerous (ranging from 1.414 – 6.286 µg/mL) as well as normal (>25 µg/mL) cell lines.

CONCLUSIONS

Marrubium vulgare L. though studied for its cytotoxicity against different cell line but was limited to aerial parts only, however this first study on roots of the herb with

promising results reveals that root extract (BuOH fraction) showing promising antioxidant and cytotoxic activities could be used in the treatment of cancer. Moreover, active compounds responsible for this activity need to be investigated as it is rich in terpenoids and phenols.

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Disclosure statement

All contributors of this manuscript declare no conflict of interest in this study.

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