Journal of King Saud University – Science 32 (2020) 2868–2879

Contents lists available at ScienceDirect

Journal of King Saud University – Science

journal homepage: www.sciencedirect.com

Original article

GCMS fingerprinting, *in vitro* pharmacological activities, and *in vivo* anti-inflammatory and hepatoprotective effect of selected edible herbs from Kashmir valley

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ARTICLE INFO

Article history:
Received 4 November 2019
Revised 12 July 2020
Accepted 15 July 2020
Available online 21 July 2020

Keywords:
GC–MS
Nutritious herbs
Melissa officinalis
Taraxacum officinale
Urtica dioica
Anticancer
Antioxidant
Hepatoprotective

ABSTRACT

The people of Kashmir customarily practice traditional medicines for curing various ailments. 3 nutritious herbs; *Melissa officinalis* L (Lamiaceae, MO), *Taraxacum officinale* L (Compositae, TO), and *Urtica dioica* L (Urticaceae, UD) were selected based on their ingestion as a folklore remedy for treating various illness, including infections, inflammation, and cancer. We aimed to scientifically validate their indigenous usage. Plant extracts were prepared by extraction in 95% methanol and subjected to qualitative phytochemical screening, total phenolic (TPC), flavonoid content (TFC), and Gas chromatography-mass spectrometry (GC–MS). *In vitro,* antioxidant and antiproliferative activities were determined. For *in vivo* study; 56 Wister rats were randomly assorted into 8 groups. Rats in the control group received saline, toxicity group received Acetaminophen/paracetamol (APAP, 2 g/kg b.w) orally for 7 days. Treatment groups received 300 mg/kg of MO, TO, or UD, respectively for 7 days after APAP (2 g/kg b.w) administration. Serum inflammation markers, antioxidant parameters, and histopathology were investigated. The GC–MS of methanol extracts indicated 16 compounds in MO (21.6% 1-nitro-β-D-arabinofuranos, as major compound), 19 compounds in TO (30.06% rutin, as major compound) and 15 compounds in UD (29.86% saponin, as major compound). TO exhibited more significant antiradical capacity in DPPH assay (IC_{50} 29.6 ± 1.12 μg/mL) and antioxidant activity in CUPRAC assay (889.34 ± 5.65 μM Trolox/g DW of extract) compared to MO (657.77 ± 5.21) and UD (534.45 ± 4.56). MO, TO and UD exhibited potent anti-proliferative potency against HT 29 and HCT 116 cancer cells, while no cytotoxicity against normal Vero cell lines. MO, TO, and UD ameliorated ($p < 0.001$) APAP-induced hepatotoxicity by improving elevated ALT, AST, and ALP levels and significantly ($p < 0.001$) decreasing TNF-α and IL-6 levels in serum. Histological examinations confirmed the biochemical findings. The present study confirmed the scientific basis for the application of (selected) medicinal herbs (studied). Plant extracts revealed antioxidant and hepatoprotective potential against APAP-induced liver injury. Further investigations to understand the mechanism of action and use in clinical trials is recommended.

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Abbreviations: GCMS, Gas chromatography-mass spectrometry; DPPH, 2, 2-Diphenyl-1-picrylhydrazyl; CUPRAC, Cupric reducing antioxidant capacity; CFU, Colony-forming unit; APAP, Acetaminophen/paracetamol; LFTs, Liver function tests; TNF-α, Tumor necrosis factor-alpha; IL-6, Interleukin 6; GSH, Glutathione; SOD, Superoxide dismutase; POD, Peroxidase; MDA, Malondialdehyde; LPO, Lipid peroxidation; DDMP, 2, 3-dihydro-2, 5-dihydroxy-6-methyl-4H-pyran-4-one; PUFAs, Polyunsaturated fatty acids; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALP, Alkaline phosphatase.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jksus.2020.07.011
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1. Introduction

Jammu and Kashmir Himalaya's often referred to as *Terrestrial Paradise on Earth*, is situated at the Northwestern tip of the Himalayan biodiversity hotspot. The valuable indigenous knowledge, when accompanied and validated by the most modern scientific insights, can propose novel drug leads (Malik et al., 2011b). For the current investigation, we have selected the three edible herbs. These herbs were chosen based on recorded ethnobotanical information, confirmation for their sustained usage, and indigenous accessibility.

Melissa officinalis L. (*Lamiaceae*) is a perennial edible herb commonly called lemon balm. In India; lemon balm is cultivated in Jammu and Kashmir, Uttrakhand, and some parts of South India (Verma et al., 2015). MO is traditionally used as an antispasmodic, anti-insomnia tonic, carminative, painkiller, for digestion, antidepressant, memory booster, antibacterial, antiviral (Verma et al., 2015). MO essential oil possesses antimicrobial, anticancer, antidiabetic, anti-inflammatory, and antioxidant effects (Moacă et al., 2018). Citronellal, citral, geranial, terpinene, rosmarinic acid, caffeic acid, several flavonoids (luteolin-7-*O*-glucoside, isoquercitrin, apigenin-7-*O*-glucoside, and rhamnocitrin), methyl carnosoate, rosmarinic acid, ferulic acid, 2 (3, 4-dihydroxyphenyl), and hydroxycinnamic acid are main metabolites identified in MO oil.

Taraxacum officinale L. (*Compositae*) commonly known as Dandelion (Local Kashmiri name: Handh) is a perennial edible herb. In Jammu and Kashmir, it is commonly distributed throughout Gurez, Tilel, Dachigam, Dubjan, Sonamarag, and Gulmarg. TO is traditionally used in the cure of various problems i.e., for the treatment of chronic cough, asthma, infection, acidity, urinary disorders, cirrhosis, jaundice, dyspepsia with constipation, and heart weakness, gout, eczema, blood purifier and specially used by women's after childbirth to prevent inflammation (Choi et al., 2010). The leaves and roots possess antitumor, antioxidant, and hypolipidemic activities. The roots contain carbohydrates, sesquiterpene lactones, carotenoids (lutein), fatty acids (myristic), flavonoids (apigenin and luteolin), and triterpenes while leaves possess cichoriin, Taraxalisin, coumarins, and aesculin (Nahid et al., 2008).

Urtica dioica L. (Urticaceae) commonly known as stinging nettle (Local Kashmiri name: Soyi) is an abundant herb that grows on moist and fertile soil. In folk medicine, it is used as a remedy for paralytic limbs or flailing arthritic, rheumatism. (Malik et al., 2011a). UD is infrequently domesticated because of its sting, however, the species is still prevalent as medicines and food in under developing countries. UD is a worthy source of caffeic acid analogs, flavonoids, and phenylpropanoids (Adhikari et al., 2016). Further work should be done to learn the medicinal value of these three edible herbs from Kashmir cultivars.

We aim to investigate the antioxidant, anticancer, hepatoprotective, and anti-inflammatory potential of three selected herbs. Furthermore, the phytochemical analysis was validated by GCMS analysis.

2. Material and methods

2.1. Plant collection

Melissa officinalis L. (MO; leaves, and stem), Urtica dioica L. (UD; leaves, and stem) and Taraxacum officinale L. (TO; leaves, stem, and roots) were collected in July from Qazigund area, Anantnag District, Jammu, and Kashmir. Plant specimens were identified by Raouf Ahmad Mir (Head Product Development, Globils Agri and Food Enterprises, Lassipora, Pulwama, J&K) and voucher specimens (#0235601, #0235602 and 0235603) were deposited at the herbarium of Globils Agri and Food Enterprises (Lassipora, Pulwama, J&K, India).

2.2. Ethics statement

No particular authorizations were required for the collection of plants as the areas were not in private-possession or secure in any way and the field studies did not encompass endangered or protected species.

2.3. Description of plant collection area

Anantnag district is in the southern sector of Jhelum Valley, geographically lies between 33°-20′ to 34°-15′ north latitude and 74°–30′ to 75°–35′ east longitude. It is the third most populous district of Jammu and Kashmir. It comprises of thick forests and mountains. The weather is mild cold in summer also. Map of the study area is shown in Fig. 1.

2.4. Plant extracts preparation

Herbs were dried in the shade for 10 days and powdered with a blender. Each plant powder (5 g) was added to 100 mL methanol (95%) and placed in a water bath at 37 °C for 3 h. The mixture was extracted twice with 100 mL of methanol for 72 h at room temperature and filtered through Whatman No. 1 filter paper. The filtrates were concentrated in vacuo using a rotary evaporator (Buchi, R114, Switzerland).

2.5. Qualitative and quantitative phytochemical screening

The extracts were subjected to qualitative phytochemical analysis by the well-defined methods for the detection of flavonoids, alkaloids, saponins, terpenoids, tannins, cardiac glycoside, reducing sugars, pholobatannins, coumarins, and anthraquinones. Total phenolic content was measured using Folin-Ciocalteu reagents. An aqueous solution of gallic acid (10–500 mg/L concentrations) was used for calibration. The results were represented as mg gallic acid equivalents (GAE)/g sample. The total flavonoid content was checked according to the colorimetric assay (Afsar et al., 2016). Aqueous solutions of known rutin concentrations in the range of 50–100 mg/L were used for calibration and the results were expressed as mg rutin equivalents (REQ)/g sample.

2.6. Gas chromatography-mass spectrometry (GC/MS)

To investigate the chemical fingerprinting of the different crude extracts, GC/MS analysis was performed using Thermo GC -Trace Ultra version 5.0, apparatus combined with Thermo MS DSQ II mass spectrometer. The mixtures were partitioned on a ZB 5-MS capillary regular non-polar column (30 m × 0.25 mm ID × 0.25 μ m FILM) to purify the samples. The column temperature was set at 70 °C for 2 min, 70–260 °C at 6 °C/min, and as a final point held for 10 min at 260 °C. The particle-free diluted sample was introduced in splitless mode (split-flow: 10 mL/min, splitless time: 1 min). The carrier gas (helium) was employed at a constant flow rate of 1 mL/min and 1 μL sample was injected. The relative percentages of crude extract constituents were quantified as peak area normalization. The mass spectral scan range was set in full scan mode from 50 to 650 (m/z). The compounds were identified by comparing their retention indices with those of authentic samples deposited on the Wiley9 and main lab computer library search program; built up using authentic compounds (Casuga et al., 2016).

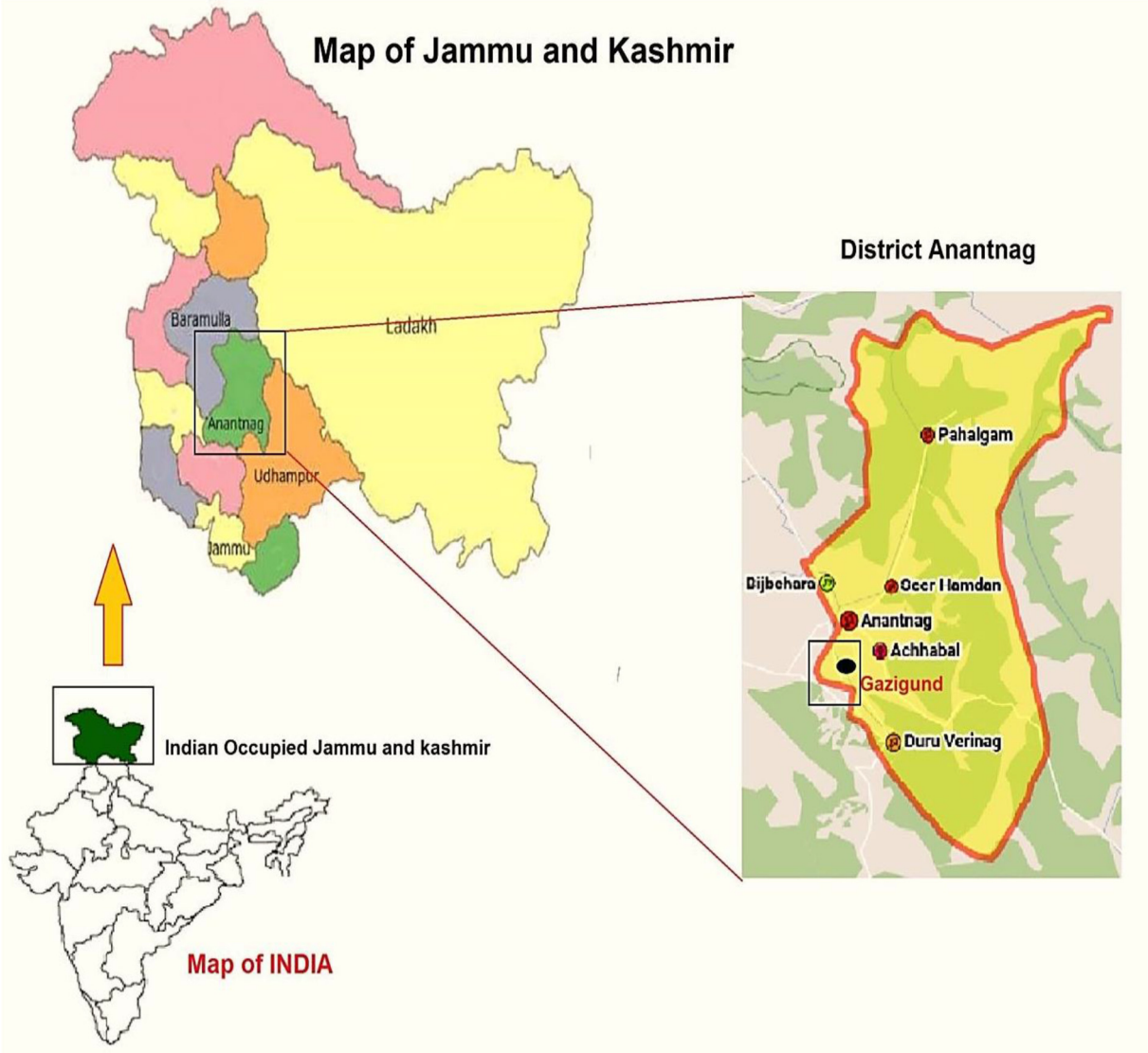

Fig. 1. Map of study area. Maps were downloaded from www.mapsofindia.com and modified.

2.7. Determination of antioxidant activity

2.7.1. Sample preparation

The stock solution of each extract was prepared in methanol (95%) at a concentration of 1 mg/mL and diluted to make the successive dilutions (0–250 μg/mL).

2.7.1.1. DPPH assay. The DPPH assay was done by the previously described protocol (Afsar et al., 2016). The absorbance was recorded using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) at 517 nm. The percentage of inhibition was assessed as follow:

$$DPPH\ scavenging\% = \frac{Abs\ of\ DPPH - abs\ of\ test\ sample}{Abs\ of\ DPPH} \times 100$$

As a standard reference compound ascorbic acid was employed.

2.7.1.2. Reducing capacity measurement using CUPRAC technique. The cupric ion reducing the antioxidant ability of all extracts was evaluated following the previously described scheme (Al-Rimawi et al., 2016). The absorbance was noted at 450 nm compared to the reagent blank. A Standard curve was set via various doses of Trolox.

The results were shown as: $\left[\frac{\mu mol\ \text{Trolox}}{g}\right]$

2.8. Anti-proliferative activity

2.8.1. Cell lines and culture conditions

Human colorectal cancer cell lines HCT 116 and HT 29 (ATCC® CCL-247™ and ATCC® HTB-38™ respectively) and purchased from American Type Culture Collection (MD, USA). A *Vero* (CCL-81™, normal kidney cells) cell line was obtained from ATCC (Manassas, VA, USA). HCT 116 and HT 29 were grown in a CO_2 (5%) atmosphere at 37 °C in medium (DMEM medium 1640 (GIBCO), 10% fetal bovine serum and 1% penicillin/streptomycin). MO, TO and UD extract samples suspended in DMSO were tested for anti-proliferative activity against both cell lines. Cells were grown to get 70% confluence and treated with different concentrations (0–50 μg) of each extracted sample for 48 h. The dilution of DMSO applied for each treatment was 0.1% (V/V).

2.8.1.1. Measurement of Anti-proliferative activity. 10×10^3 cells/well were grown in 1 mL of culture medium comprising 0–250 μg dilution of each sample in 96-well plate. Cells were incubated for 48 h at 37 °C, 200 μL of 3–4,5-dimethylthiazol-2-yl)-2,5 -diphenyl tetrazolium bromide (5–7 mg/mL PBS) was added to each well and incubated for 2 h and 200 μL of DMSO was added and spun (1800×*g* for 5 min at 4 °C). The absorbance was taken at 540 nm on a microplate reader (Elx 800). Cell viability in each tested sample was calculated by the following formula:

$$\textit{cell viability} = \left[\frac{\textit{Abs of sample} - \textit{abs of blank}}{\textit{Abs of control} - \textit{abs of blank}}\right] \times 100$$

$$\text{Cell death}(\%) = 100 - -\%\,\text{Cell survival}$$

Blank is the absorbance of only cell culture media without the cancer cells.

IC_{50} values were calculated using Graph pad prism 5

2.9. Animal model of hepatotoxicity

Adult male Wister rats, weighing 200 – 220 g, were kept under standard laboratory conditions on a 12-h light/dark cycle at 25° C ± 2° C. The animals had free access to standard rat pellet diet with water *ad libitum*. All animals were kept following the recommendations of the guidelines for the care and use of laboratory animals (NIH publications no. 80–23; 1996).

2.9.1. Acute toxicity testing

The acute toxicity examination was led in line with the guideline 425 of the Organization for Economic Cooperation and Development (OECD) for the analysis of substances for acute oral toxicity. Male Wister rats (n = 6) were treated with different doses (50, 250, 500, 1000, 2000, and 3000 mg/kg, p.o.) of MO, TO and UD, while the control group received distilled water. Animals were detected constantly for 2 h for comportment, and autonomic profiles and later 24 h and 72 h for any fatality (Afsar and Razak, 2019).

2.9.2. In vivo Experimental plan

56 male Wister rats were randomly allocated into 8 groups (n = 7). The group I received saline. Group II, III, and IV were treated orally for 7 days with 300 mg/kg dose of MO, TO and UD respectively. In Group V received Acetaminophen/paracetamol (APAP) (2 g/kg b.w) for 7 days orally (Mishra et al., 2015). Group VI, VII, and VIII were co-treated with APAP + MO (300 mg/kg b.w, oral), APAP (2 g/kg b.w) + TO (300 mg/kg b.w, oral) and APAP (2 g/kg b.w) + UD (300 mg/kg b.w, oral) respectively for 7 days.

24 h after the last treatment, all animals were weighed and sacrificed by cervical dislocation. Trunk blood was taken and centrifuged at 500×*g* for 15 min at 4 °C to obtain serum and kept on −80 °C for further analysis. The liver was removed; one-half was preserved in liquid nitrogen and stored at −80 °C for further biochemical analysis while the remaining half was processed for histology.

2.9.3. Liver function tests (LFTs) in serum

Serum examination of various liver function biomarkers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) was estimated by using standard AMP diagnostic kits (Stattogger Strasse 31b 8045 Graz, Austria).

2.9.4. Determination of serum TNF-α and IL-6

Concentrations of TNF-α and IL-6 in serum were determined using ELISA kits according to the manufacturer manual (R&D systems).

2.9.5. Estimation of tissue protein content

Total soluble protein content within the tissue samples was estimated using a previously established protocol (Afsar and Razak, 2019).

2.9.6. Assessment of biochemical parameters

GSH, SOD, and MDA levels in liver tissues were tested following previously described protocols (Afsar and Razak, 2019).

2.9.7. Histopathological examination by light microscopy

Liver tissues from the respective groups were preserved in buffered formalin. After dehydration tissue samples were secured in paraffin to make blocks for microtomy. Tissues were sectioned 4–5 μm with a microtome and stained with Hematoxylin-Eosin (H&E) and studied under a light microscope (DIALUX 20 EB) at 40X.

2.9.8. Statistical tests

All the experiments were carried out in triplicate. Results were expressed as mean ± standard error of the mean (SEM). One-way and two-way ANOVA were used for statistical analysis using Graphpad Prism software. P values of <0.05 were considered significant.

3. Results and discussion

3.1. Qualitative and quantitative phytochemical screening

Table 1 shows the qualitative and quantitative phytochemical analysis of methanol extract of MO, TO, and UD. Qualitative phytochemical screening indicated various polar and nonpolar components in all extracts. The chemical composition of MO oils has done previously (Pirbalouti et al., 2019); however, this is the first report on the methanol crude extract collected from Kashmir. Phenolic and flavonoid constituent function as antioxidants and play a role in combating cancer, infection, and vast degenerative infirmities (Afsar et al., 2016). The quantity of phenolic and flavonoid content in MO, TO, and UD methanol extracts were calculated from the standard calibration curve of gallic acid (R^2 = 0.93) and rutin (R^2 = 0.92) respectively. TO showed the highest content of TPC and TFC, followed by MO and UD (Table 1). The total phenolic contents of TO extract determined in the current study were noted to be higher than calculated from methanol extract of Brazilian species (Colle et al., 2012). This might be linked to variation in agro-climatic conditions, maturity at harvest as well as a difference in extraction technique and polarity of extracting solvent.

3.2. Compound fingerprinting by GCMS

3.2.1. Chemical profiling of M. officinalis

GCMS chromatogram detected the presence of 16 constituents in the methanol extract of MO harvested in Kashmir (Fig. 2a, Table 2), representing 91.2% of the composition of MO. Dominant compounds on the basis of % peak area and bioactivity were 1-nitro-β-D-*arabino*-furanos (21.57%), 3-hydroxy-2 methyl-4 h-pyran-4-one (maltol, 11.54%), methyl (E)-3-acetoxy-4-nitro-2-butenoate (9.71%), 2,3-dihydro-3,5-dihydroxy-6-methyl-4 h-pyra n-4-one (DDMP, 8.54%), decanoic acid (7.21%), DL-glyceraldehyde dimer (6.08%), 2-furan-carboxaldehyde (5.9%), 4-methyl-morpholine (2.93%). Furan and pyran derivatives are major compounds identified in MO by GCMS possess anticancer, antioxidant, and antimicrobial potentials (Chai et al., 2013). while DDMP is a saponin with proven antioxidant and antitumor potentials (Čechovská et al., 2011). The presence of bioactive metabolites in MO might be attributed to the therapeutic actions of MO.

Table 1
Qualitative and quantitative phytochemical screening and antioxidant activity of *Melissa officinalis* (MO), *Taraxacum officinale* (TO), *and Urtica dioica* (UD) methanol extracts.

Tests	Samples			
	Melissa officinalis (MO)	Taraxacum officinale (TO)	Urtica dioica (UD)	Ascorbic acid (ASC)
Qualitative phytochemical screening				
Tannins	+	+	+	N/A
Steroids	+	+	+	N/A
Saponins	+	+	+	N/A
Alkaloids	+	+	+	N/A
Flavonoids	+	+	+	N/A
Coumarins	+	+	–	N/A
Terpenoids	+	+	+	N/A
Pholobatanins	+	+	–	N/A
Reducing sugars	+	+	+	N/A
Anthraquinones	+	–	–	N/A
Cardiac Glycosides	+	+	+	N/A
Quantitative phytochemical screening				
TPC (mg gallic acid equivalent/g dry sample	151.6 ± 1.21	185.3 ± 1.15**	99.5 ± 1.98***,##	N/A
TFC (mg rutin equivalent/g dry sample	117 ± 3.52	149 ± 1.32**	84 ± 1.04***,##	N/A
Antioxidant activity				
DPPH IC50 (μgml)	43.8 ± 1.22	29.6 ± 1.12***	76.3 ± 1.92***,+++,###	25.4 ± 1.51
CUPRAC (μmol Trolox/g DW of extract)	657.77 ± 5.21	889.34 ± 5.65***	534.45 ± 4.56+++,###	

A negative sign (–) indicates absence, positive sign (+) indicates presence, N/A indicates not applicable for the specific testing. Values are expressed as mean ± SEM (n = 3). Data analyzed by One-way ANOVA using Tukey's Multiple Comparison Test. Asterisks *, **, *** represent significance at p < 0.01 and p < 0.0001 from MO, + represent significance at p < 0.01 from TO and #, ### represent significance at p < 0.01 and p < 0.0001 from ASC standard compound. MO: Melissa officinalis, TO: *Taraxacum officinale and* UD: Urtica dioica.

3.2.2. Chemical profiling of T. officinalis

GCMS analysis detected 19 compounds in the methanol extract of TO harvested in Kashmir (Fig. 2b, Table 3) representing 99.99% of the TO compositions. Dominant active compounds on the basis of % peak area and retention time were hydroxy-benzeneacetic acid (rutin metabolite, 30.060%), β-amyrin (10.790%), eicosane (8.580%), Lup-20(29)-en-3-ol (7.320%), hentriacontane (6.54%), 3-methyl-2-pentanone (6.24%), hexadecanoic acid (3.160%), methyl ester of hexadecanoic acid (2.78%), lupeol (2.99%), and tritetracontane (2.10%). Active metabolites detected in TO belongs to phenols, terpenes, fatty acids, and alkanes classes. The identification of triterpenes in TO is scientific validation of its use in inflammatory conditions, especially after pregnancy because the identified compounds have potent anti-inflammatory activity. The antioxidant action of TO might be attributed to amyrin isomers. β-amyrins have anti-apoptotic, antioxidant, anti-inflammatory, anti-fibrotic, and hepatoprotective effects (Ghosh et al., 2015). The bioactive fatty acids identified in TO are palmitic acid (methyl ester of hexadecanoic acid 2.780% and hexadecanoic acid 3.160%). Palmitic acid possesses antimicrobial, antitumor, and antioxidant activities (Harada et al., 2002; Pinto et al., 2017). Lupeol has diverse therapeutic potentials including antioxidant, anticancer, hepatoprotective, chemo-preventive, and anti-inflammatory (Wal et al. 2011).

3.2.3. Chemical profiling of U. dioica

GCMS analysis detected 15 compounds in the methanol extract of UD harvested in Kashmir (Fig. 2c, Table 4) representing 95.38% of UD chemical composition. Dominant active compounds identified on the basis of % peak area and bioactivity were 2,3-dihy dro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one (29.860%), β-ketoglutaric acid (15.910%), N-(phenethyl) phenylacetamide (15.260%), 3-amino-2-oxazolidinone (7.530%), α.-L-galactopyranoside (5.380%), Ketoglutaric benzenepropanoic acid/hydrocinnamic acid (4.570%), hexadecanoic acid (4.250%), and 9, 12-octadecadienoic acid (2.020%). In UD, the major antioxidant and anti-mutagenic components identified are 2, 3-dihydro-3, and 5-dihydroxy-6-methyl-4 h-pyran-4-one (DDMP). The previous report revealed DDMP as a major antioxidant compound in prunes and plums (Čechovská et al., 2011). α-ketoglutarate is the main component of the Krebs cycle that plays a role in protein synthesis, bone development and not only extends lifespan but also delays age-related diseases, indicating its role in the prevention and treatment of aging and age-related diseases (Wu et al., 2016). Linolenic acids are essential long-chain polyunsaturated fatty acids (PUFAs) that decrease chronic degenerative and inflammatory diseases (Saha and Ghosh 2012). The therapeutic potential of combine fatty acid composition may probably contribute to the health benefits of UD.

3.3. Determination of antioxidant activity

The free radical scavenging potential of MO, TO and UD extracts against DPPH radical in comparison with standard antioxidants ascorbic acid is shown in Fig. 3a. The IC_{50} values of all tested extracts and standard antioxidants are shown in Table 1. The overall activity followed the sequence; ascorbic acid > TO > MO > UD. The observed anti-radical activity would be attributed to the highest amount of phenolic and flavonoids in these herbal extracts. Correlation analysis indicated that the DPPH radical quenching activity of extracts showed good correlation with TPC (R^2 = 0.9879) and TFC (R^2 = 0.8477).

The CUPRAC absorbance of the incubation solution due to the reduction of Cu (II)-neocuproine reagent decreased in the presence of polyphenolics. The extracts (250 μg/mL) exhibited good CUPRAC total capacities. The reducing potential of studied herbal extracts was in the order of TO > MO > UD (Table 1). Reducing the potential of the methanol extracts of tested herbal extracts showed a good correlation with total phenolic (R^2 = 0.979) and flavonoid content (R^2 = 0.8977). The Significant antioxidant potential of the individual herb was observed by other researchers while examining the antioxidant activity of TO flower extract (Colle et al., 2012), ethanol, and water extract of MO leaves (Koksal et al., 2011) and protein fractions from aerial parts of UD (Di Sotto et al., 2015). Secondary metabolites have been identified in all extracts by GCMS that have proven antioxidant activities.

3.4. Specific anti-proliferative activity against cancer cells

The result of the MTT assay revealed that all extracts inhibited colon cell proliferation in a dose-dependent response; maximum

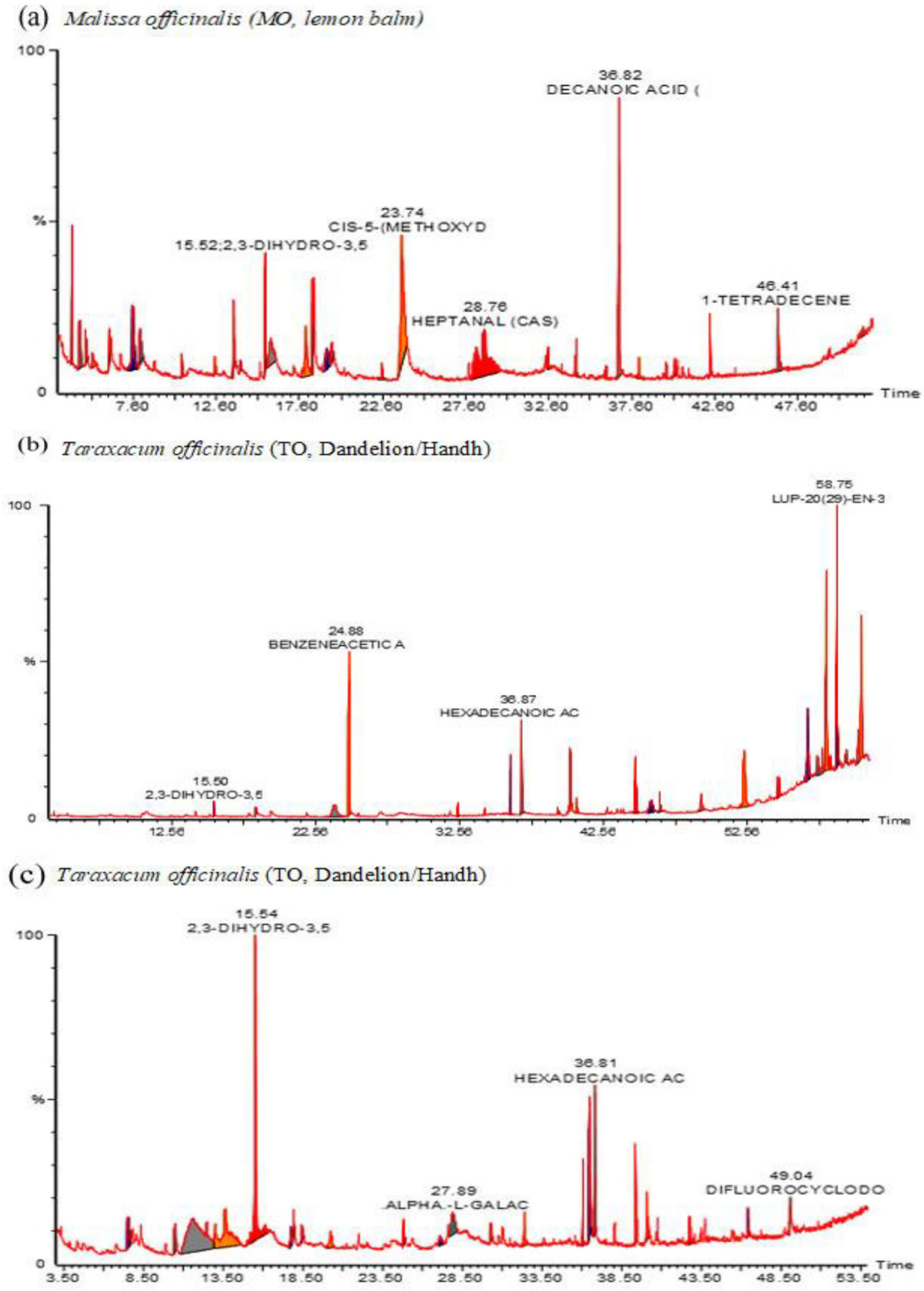

Fig. 2. GCMS fingerprinting of crude methanol extract of *Malissa officinalis (MO, lemon balm)*, *Taraxacum officinalis* (TO, Dandelion/Handh), and *Urtica dioica (UD, Nettle/soyi)*. GCMS analysis detected 16 compounds in MO, 19 compounds in TO, and 15 compounds in UD. Compounds were identified based on % peak area and retention time.

cell inhibition was observed at 250 μg/mL against both cell lines (Fig. 3b and c). UD treatment was more effective against both cell lines compared to MO and TO, with IC_{50} values found to be 35.30 ± 1.86 μg/mL for HT 29 cell line, and 49.80 ± 1.85 μg/mL for HCT 116 cell line (Table 5). All tested extracts did not show growth inhibitory effects against normal *Vero* cells up to 250 μg/mL. The anti-cancer potential was in the order of UD > TO > MO. In a previous investigation, 50% ethanolic extracts of MO showed significant inhibition of cell proliferation against HTC116 cells after 72 h of treatment, reducing cell proliferation to values close to 40% at 5 μg/mL dose (Encalada et al., 2011). The furans and pyran metabolites seem to be the bioactive, involved in inducing anticancer

Table 2
Compounds identified in *Melissa officinalis* (MO) through GCMS.

Peak	Compounds	RT	Area	% Area	Compound class/activity
1	METHYL (E)-3-ACETOXY-4-NITRO-2-BUTENOATE	7.55	103,097	9.71	acrylate ester
2	DL-GLYCERALDEHYDE DIMER	7.99	64,618	6.08	Carbohydrate (preservative)
3	2,4-DIHYDROXY-2,5-DIMETHYL-3 (2H)-FURAN-3-ONE	10.52	15,355	1.450	Furan (Aroma compound/antioxidant/ anticancer)
4	4-METHYL-MORPHOLINE	12.51	31,164	2.930	Tertiary amine (Pharmaceutical industry/ Antibacterial, anticancer)
5	3-HYDROXY-2-METHYL-4H-PYRAN-4-ONE	13.63	122,546	11.54	Phenol/Maltol (antioxidant, anticancer (Wang, Jenner et al. 2007)
6	CYCLOBUTANOL	14.04	48,513	4.570	Cyclic alcohol
7	2,3-DIHYDRO-3,5-DIHYDROXY-6-METHYL-4H-PYRAN-4-ONE	15.52	89,778	8.450	(DDMP) (anticancer, antioxidant (Čechovská et al., 2011)).
8	2-FURANCARBOXALDEHYDE	17.96	63,426	5.970	Aldehyde/Furfural (Major flavor component, antimicrobial (Chai et al., 2013)
9	1-NITRO-.BETA.-D-ARABINOFURANOS	18.41	229,129	21.570	heterocyclic compounds Nucleoside (antineoplastic)
10	2,3-DIHYDROXY-PROPANAL	19.22	54,504	5.130	Glyceraldehyde metabolite
11	HEPTANAL	28.76	19,636	1.85	Alkyl aldehyde (flavoring agent)
12	DECANOIC ACID	36.82	76,636	7.210	Fatty acid /Capric acid Flavoring agent, anticancer activity on cultured human colorectal, skin and breast cancer (Narayanan, Baskaran et al. 2015)
13	ISOPROPYL MYRISTATE	38.04	5897	0.560	Ester of isopropyl alcohol and myristic acid
14	EICOSYL ACETATE	40.24	9443	0.890	Fatty ester/pheromone
15	BETA.-H-PREGNANE	42.31	18,559	1.750	steroid
16	TETRADECENE	46.41	23,985	2.260	Acyclic olefins.

Table 3
Compounds identified in *Taraxacum officinalis* (TO) through GCMS.

Peak	Compounds	RT	Area	Area %	Compound class/activity
1	2,3-DIHYDRO-3,5-DIHYDROXY-6-METHYL 4-H-PYRAN-4-ONE	15.50	25,150	0.440	Saponin/DDMP (Antioxidant, anticancer (Čechovská et al., 2011))
2	3-HYDROXY-HEXANOIC ACID	18.39	137,126	2.410	Fatty acid
3	3-METHYL-2-PENTANONE	23.90	354,832	6.240	Aliphatic ketone (flavoring agent)
4	HYDROXY-BENZENEACETIC ACID	24.88	1,710,578	30.060	Phenol/Rutin metabolite (antioxidant anticancer against colon cancer (Amić et al., 2016)
5	TETRADECANOIC ACID	32.44	25,427	0.450	Fatty acid/Myristic acid (anticancer, Reno-protective)
6	METHYL ESTER OF HEXADECANOIC ACID	36.09	158,163	2.780	Fatty acid/palmitic acid methyl ester/metabolite (antimicrobial, antitumor, antioxidant (Harada et al., 2002, Pinto et al., 2017)
7	HEXADECANOIC ACID	36.87	179,928	3.160	Fatty acid /palmitic acid (antimicrobial, antitumor ,antioxidant (Harada et al., 2002, Pinto et al., 2017))
8	DECANENITRILE	40.26	124,901	2.190	Nitrile (fragrance)
9	4-ETHYL-2,6-DIMETHTHYL-4-HEPTANOL	40.69	18,395	0.320	Alcohol (Sweat aroma)
10	HENTRIACONTANE	44.79	372,386	6.540	Alkane (anti-inflammatory, antioxidant, antimicrobial, anticancer (Khajuria, Gupta et al. 2017)
11	2-HYDROXY-1-HEXADECANOIC ACID	46.47	37,435	0.660	Fatty acid
12	TRITETRACONTANE	49.33	119,447	2.100	Alkyl (Anti-inflammatory)
13	NONACOSANE	52.31	45,389	7.980	Alkane/ plant metabolite (Pheromone)
14	2-OCTYLDODECAN-1-OL	54.70	157,603	2.770	Fatty Alcohol (emollient)
15	EICOSANE	56.71	488,368	8.580	Alkanes (plant metabolite, antioxidant, Anti-tumor activity against the human gastric SGC-7901 cell line (Sivasubramanian and Brindha 2013)
16	COPROSTAN-16.BETA.-OL	57.74	126,612	2.220	Cholesterol
17	BETA.-AMYRIN	58.03	614,141	10.790	Pentacyclic triterpenoid (Anti-inflammatory, antimicrobial, antioxidant (Ghosh et al., 2015))
18	LUP-20(29)-EN-3-OL	58.76	416,300	7.320	Triterpene (antioxidant, anticancer, hepatoprotective, antibacterial, chemo- preventive, anti-inflammatory (Wal, Wal et al. 2011))
19	LUPEOL	60.44	170,112	2.990	Triterpene (Wal, Wal et al. 2011)

activity in MO. UD has been used traditionally for cancer treatment and this is the first scientific validation of the anti-proliferative potential of UD against colon cancer cells. In TO, the major compound detected by GCMS examination was Rutin metabolite (30.06%) besides several other constituents with potent anticancer and anti-inflammatory potential (Amić et al., 2016). The major anticancer metabolite in UD seems to be saponin (DDMP: 29.86%). DDMP significantly suppresses cancer growth in colon cell lines (Salyer 2011). Anti-Proliferative effects of tested herbs seem to be cell type-specific, as extracts did not show growth inhibitory effects against normal *Vero cell* lines at tested doses.

3.5. In vivo anti-inflammatory and hepatoprotective effect

Acetaminophen or paracetamol (APAP) is a frequently used antipyretic and analgesic drug. The APAP-induced toxicity model is generally used to investigate the prospective hepatoprotective action of plant extracts/compounds.

3.5.1. Acute toxicity evaluation

MO, TO and UD were found to be safe at all tested doses (up to 3000 mg/kg b.w) and did not induce any detrimental indications in rats like sedation, convulsions, diarrhea, and irritation. During the 72 h of assessment, no mortality was observed. Therefore, one-

Table 4
Compounds identified in *Urtica dioica* (UD) through GCMS.

Peak	Compounds	RT	Area	Area %	Compound class/activity
1	B-KETOGLUTARIC ACID	7.57	384,553	15.910	Carboxylic acid (antibacterial)
2	2,3-DIHYDRO-3,5-DIHYDROXY-6-METHYL-4H-PYRAN-4-ONE	15.54	721,644	29.860	Saponin/DDMP (antioxidant, Anti- proliferative, pro-apoptotic against colon cancer, antioxidant (Čechovská et al., 2011)
3	HEXANAMIDE	17.76	33,834	1.400	Amide
4	3-AMINO-2-OXAZOLIDINONE	18.45	182,010	7.530	Metabolite of Furazolidone and Nitrofuran, (Antibacterial , antibiotic (Cooper, Elliott et al. 2004).
5	N-METHYL-3-ETHOXYAMPHETAMINE	20.25	14,373	0.590	alkaloid
6	BENZENEPROPANOIC ACID	24.82	110,513	4.570	Carboxylic acid / hydrocinnamic acid (used for flavoring, a preservative to maintain the original aroma quality of frozen foods, antioxidant, antimicrobial (Sova 2012).
7	ALPHA.-L-GALACTOPYRANOSIDE	27.86	129,912	5.380	Carbohydrate (an energy source for the body)
8	DECANOIC ACID	32.40	21,282	0.880	Fatty acid
9	PENTADECANOIC ACID	36.06	94,085	3.890	Fatty acid/milk fat
10	N-(PHENETHYL) PHENYLACETAMIDE	36.46	368,724	15.260	Amide(anticancer, anti-inflammatory, analgesic, antibacterial (Rani, Pal et al. 2014))
11	HEXADECANOIC ACID	36.81	102,593	4.250	Fatty acid/ palmatic acid (Harada et al., 2002; Pinto et al., 2017)
12	9,12-OCTADECADIENOIC ACID	39.34	48,857	2.020	Fatty acid/linoliec acid (antioxidant, antitumour (Saha and Ghosh 2012))
13	D6-DODECENE-1-OL	40.05	55,273	2.290	Acyclic Alkenes
14	2-HEXYLALLYL ALCOHOL	42.75	35,179	1.460	Alcohol
15	HEXADECANOIC ACID	46.29	2080	0.090	Fatty acid

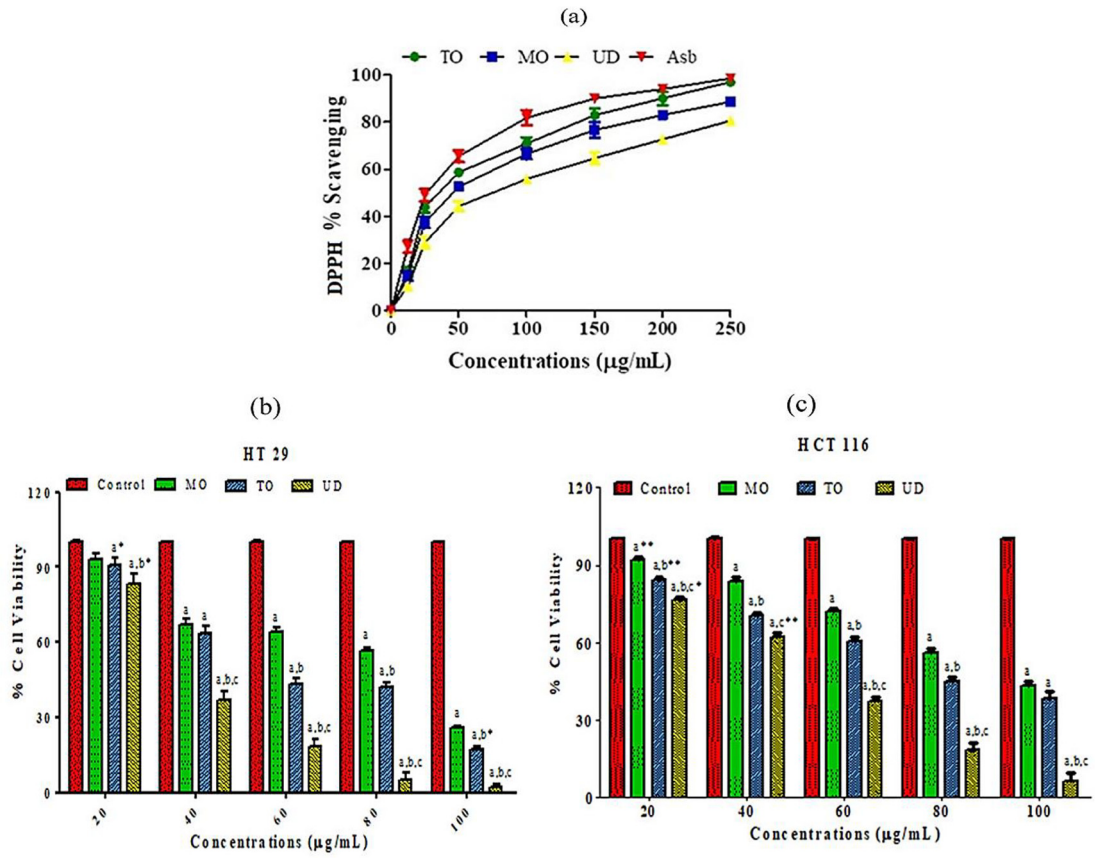

Fig. 3. (a) Dose-dependent scavenging activity of methanol extracts of 3 edible herbs (MO, TO and UD). Values are expressed as mean ± SEM ($n = 3$). GA: Ascorbic acid used as a standard reference compound. (b) The anti-proliferative potential of MO, TO and UD against HT 29 cells. (c) The anti-proliferative potential of MO, TO and UD against HCT 116 cells. Cells viability percentage measured by MTT assay. Values expressed as mean ± SEM. Alphabets show significance from the control group at $p < 0.0001$, "b" indicates significance from MO treated group at $p < 0.0001$, and "c" indicates significance from TO treated group at $p < 0.0001$. Asterisks *, ** shows significance at $p < 0.05$ and $p < 0.001$. (Two-way ANOVA accompanied by Bonferroni posttests). MO: *Melissa officinalis, TO: Taraxacum officinale and UD: Urtica dioica.*

tenth of the maximum dose, 300 mg/kg b.w. was used for *in vivo* evaluations.

3.5.2. Effect of herbal extracts on hepatic markers in serum

Administration of APAP in rats by oral route caused liver damage as indicated by a significant increase in serum enzymes ALP, AST, and ALT activity as compared with the control group (Fig. 4 I; a, b, and c). The increase in AST and ALT levels may be due to increased LPO. Co-administration of rats with MO, TO and UD extracts with APAP significantly restored the hepatic marker levels in serum towards normal values. A significant increase in serum activities of AST and ALT considered as marker enzymes of hepato-

Table 5
In vitro Cytotoxic effect of extracts treatment against HT 29 and HCT 116 cell lines and *in vivo* effect of various treatments on liver tissue antioxidant status and oxidative stress markers against APAP induced hepatotoxicity.

In vitro anti-proliferative activity			*In vivo antioxidant and oxidative stress biomarkers*				
Groups	HT 29 IC50 (μg/ml)	HCT 116 IC50 (μg/ml)	Protein (μg/mg Tissue)	MDA (nM/min/mg protein)	GSH (μM/gtissue)	SOD (U/mg protein)	POD (U/min)
Saline	N/A	N/A	4.66 ± 0.05	3.5 ± 0.156	19.98 ± 0.28	1.55 ± 0.06	13.40 ± 0.23^{b}
MO	84.50 ± 2.43	88.40 ± 2.54	4.68 ± 0.04^{+++}	3.47 ± 0.112^{+++}	20.08 ± 0.28^{+++}	1.56 ± 0.07^{+++}	13.41 ± 0.23^{+++}
TO	53.25 ± 2.11***	73.90 ± 2.36**	4.71 ± 0.15^{+++}	3.45 ± 0.087^{+++}	20.11 ± 0.16^{+++}	1.54 ± 0.08^{+++}	13.44 ± 0.27^{+++}
UD	35.30 ± 1.86$^{***,++}$	49.80 ± 1.85$^{***,+++}$	4.67 ± 0.13^{+++}	3.51 ± 0.504^{+++}	18.38 ± 0.29^{+++}	1.53 ± 0.05^{+++}	13.42 ± 0.25^{+++}
APAP	N/A	N/A	1.19 ± 0.05***	9.97 ± 0.337***	10.08 ± 0.28***	0.39 ± 0.03***	7.23 ± 0.39***
APAP + MO	N/A	N/A	4.53 ± 0.11^{+++}	4.75 ± 0.107$^{*,+++}$	17.34 ± 0.11^{+++}	1.31 ± 0.04$^{*,+++}$	12.57 ± 0.44
APAP + TO	N/A	N/A	4.61 ± 0.16^{+++}	4.14 ± 0.06^{+++}	18.32 ± 0.19^{+++}	1.35 ± 0.03$^{*,+++}$	12.50 ± 0.66
APAP + UD	N/A	N/A	4.52 ± 0.13^{+++}	4.95 ± 0.09$^{**,+++,/}$	15.83 ± 0.21$^{*,+++}$	1.29 ± 0.05$^{*,+++}$	9.21 ± 0.29$^{**,+++,\#,/}$

Values are expressed as mean ± SEM (n = 3 for in vitro anti-proliferative activity, and n = 7 for *in vivo* testing). For in vitro activity Asterisks ** and *** represent significance difference from MO group at $p < 0.001$ and $p < 0.0001$, $^{++}$ and $^{+++}$ represent significance difference from TO group at $p < 0.001$ and $p < 0.0001$ respectively. For *in vivo* experiment asterisks $^{***,\ **,\ *}$ represents significance at $p < 0.0001$, $p < 0.001$ and $p < 0.05$ *vs.* control group. $^{+++}$ Represents significance at $p < 0.0001$ *vs.* APAP group. # represents significance at $p < 0.05$ *vs.* APAP + MO group. $^{/}$ Represents significance at $p < 0.05$ *vs.* APAP + TO group. Non-significant difference ($p > 0.05$) was recorded between control and extract alone (MO, TO and UD) treated group in all parameters (One-way ANOVA followed by Tukey's multiple comparison tests). MO: Melissa officinalis, TO: *Taraxacum officinale and* UD: Urtica dioica.

cyte cytolysis. Our results are in line with previous findings that demonstrated the ameliorating potential of MO against Malathione induce alterations in hepatic function markers (Sief et al., 2015).

3.5.3. Effect of herbal extracts on pro-inflammatory biomarkers in hepatic tissue

A more evident approach to assess inflammation is to measure the extent of circulating cytokines. Therefore, the extract/compound exerting an anti-inflammatory activity might also demonstrate hepatoprotective activity. Induction of hepatotoxicity by APAP significantly ($p < 0.0001$) increased serum levels of TNF-α and IL-6 compared to control and extract alone treated groups (Fig. 4II; a, and b). Our results are similar to reports of James and coworkers suggested that TNF-α and interleukins are released in response to APAP intoxication and are responsible for certain pathological manifestations of APAP-induced hepatotoxicity (James et al., 2005). Among the tested herbs, TO showed a more significant reduction in TNF-α and IL6 levels compared to MO ($p < 0.05$) and UD ($p < 0.001$) respectively. The ability of tested herbs specifically TO to inhibit inflammatory cytokine production might be associated with its benefits in the treatment of various inflammatory conditions.

3.5.4. Measurement of oxidative stress markers and antioxidants in the liver

The combination of hepatoprotective and antioxidant activity synergistically prevents the initiation and progression of hepatocellular injury. We observed that APAP treatment resulted in substantial ($p < 0.0001$) lessening in liver tissue soluble protein as compared to control and MO, TO and UD alone treated groups (Table 5). Co-treatment with MO, TO and UD significantly ($p < 0.0001$) restored tissue protein content in comparison to APAP alone treated group. Oxidative stress has been reflected as one of the underlying mechanisms of APAP-persuaded acute organ injury. APAP-induced significant ($p < 0.0001$) intensification in MDA levels as compared to control and extracts alone treated groups. Co-treatment with MO, TO and UD resulted in a diminution in the MDA levels. MO and TO administration seems to be more effective in reducing the APAP-induced oxidative trauma compared to UD. Besides that, previous findings endorsed MO, TO and UD antioxidant activity (Koksal et al., 2011; Colle et al., 2012). The ability of MO and TO exert hepatoprotective activity possibly via its antioxidant action.

3.6. Histopathological examination

Histopathological observations demonstrated that the control group showed normal lobular architecture and hepatic cells with intact cytoplasm and well-defined sinusoids (Fig. 5) The section of APAP intoxicated liver, exhibited massive necrosis, presence of hemorrhage, and inflammation with infiltration of lymphocytes. Interestingly, these pathological changes were found to be reduced with treatment with MO, TO and UD indicating the extract's ability to reverse the APAP-induced hepatic injury. The presence of marked necrosis, hemorrhage, and inflammation following treatment with APAP was shown in other studies as well (Mahmood et al., 2014). TO seems to be more protective in preventing liver damage. Histological study apprehends our biochemical findings. MO, TO and UD persuaded hepatoprotective action is proposed to conceivably implicate the synergistic actions of flavonoids, saponins, terpenoids, and tannins (Le Tran et al., 2002; Pan and Lai, 2010).

4. Conclusion

Present study provided scientific proof for the antioxidant, anticancer, and hepatoprotective activities of MO, TO and UD. The occurrence of several bioactive compounds validates their use for the cure of various ailments by traditional practitioners and conveys supportive data for future studies that will lead to their consumption in cancer, oxidative stress, and anti-inflammatory remedies. In-depth examinations are in progress to conclude the possible hepatoprotective mechanism (s) involved and to isolate and identify the responsible bioactive compounds from the tested herbal extracts. However, anticancer potential of MO, TO, and UD warrants further examinations as a potential nutraceutical or functional food for cancer prevention.

5. Limitations of the study

Future studies are needed to confirm the findings obtained in our study using molecular tools and immune-histochemical techniques. Additionally, due to lack of funding presently we were unable to analyze the details of the signaling mechanism involved in the anti-inflammatory and anticancer potential of selected herbs. Therefore, investigating the detailed mechanism of protection and how these edible herbs induce various pharmacological

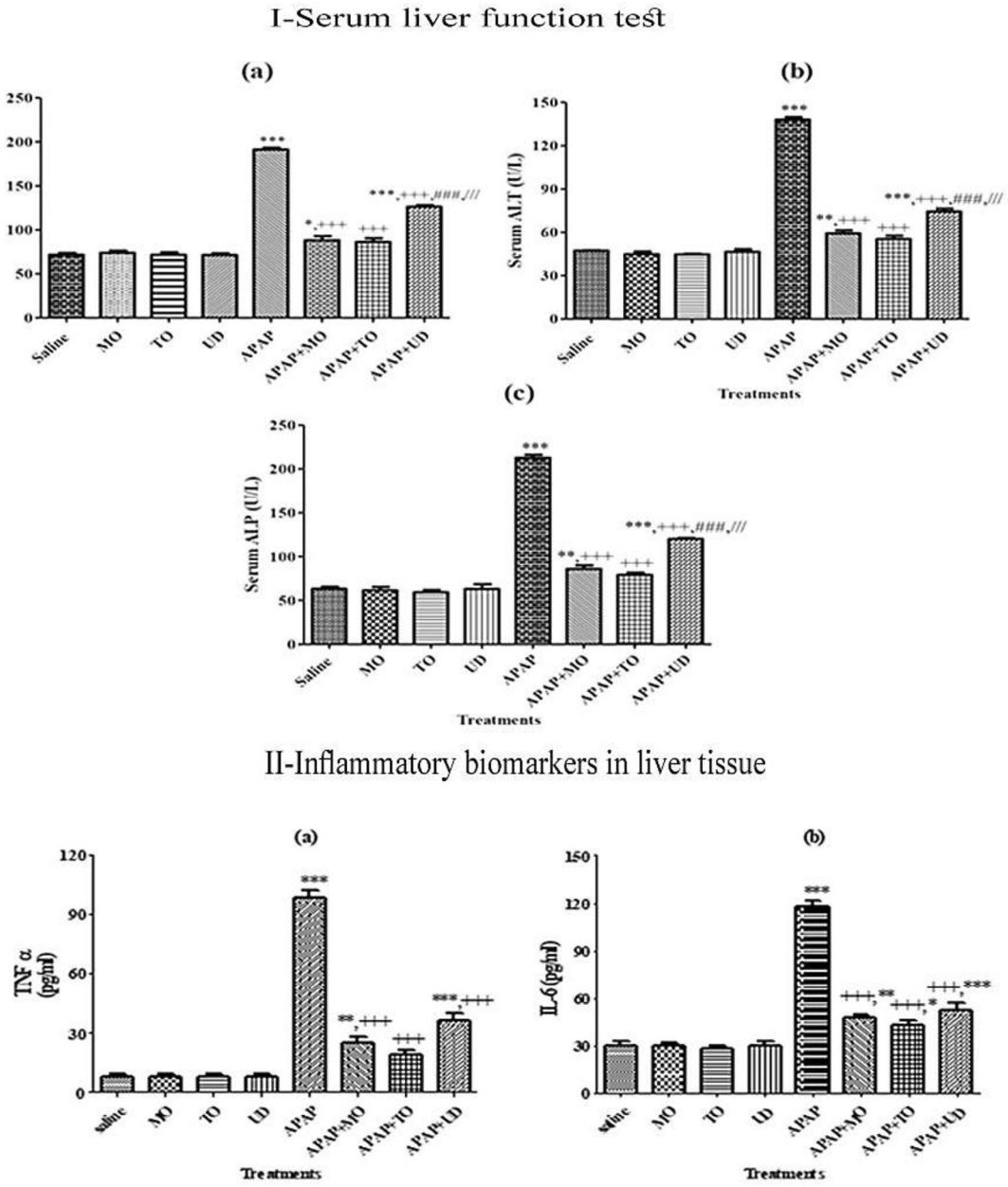

Fig. 4. I Effect of MO, TO and UD on serum liver function tests (LFTs). II: Effect on inflammatory biomarkers in Liver tissues of various treatment groups. Values are shown as Mean ± SEM (n = 7). *, **, *** indicate significance *vs*. saline group at $p < 0.05$, $p < 0.01$ and $p < 0.0001$ probability level, +, ++, +++ indicate significance from the APAP group at $p < 0.05$, $p < 0.01$ and $p < 0.0001$, ### represents significance at $p < 0.0001$ *vs*. APAP + MO group. /// Represents significance at $p < 0.0001$ *vs*. APAP + TO group. Non-significant difference ($p > 0.05$) was recorded between control and extract alone (MO, TO and UD) treated group in all parameters (One-way ANOVA followed by Tukey's multiple comparison tests). APAP: Acetaminophen/paracetamol, MO: *Melissa officinalis,* TO*: Taraxacum officinale and* UD*: Urtica dioica.*

activities with special focus on the anticancer potential in tumor models should be the focus of further investigations.

6. Declarations

6.1. Ethical approval and consent to participate

Ethical approval (# 0236) was taken from the Experimental Animal Care Committee, Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

Not applicate

6.2. Consent for publication

Not applicable

6.3. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

6.4. Authors' contributions

SR and TA made significant contributions to conception, design, experimentation, acquisition, and interpretation of data and writing of the manuscript. DA, AA, MA, AAA, and RAC made a substantial contribution to experimentation and revising the manuscript for intellectual content. All authors read and approved the final manuscript.

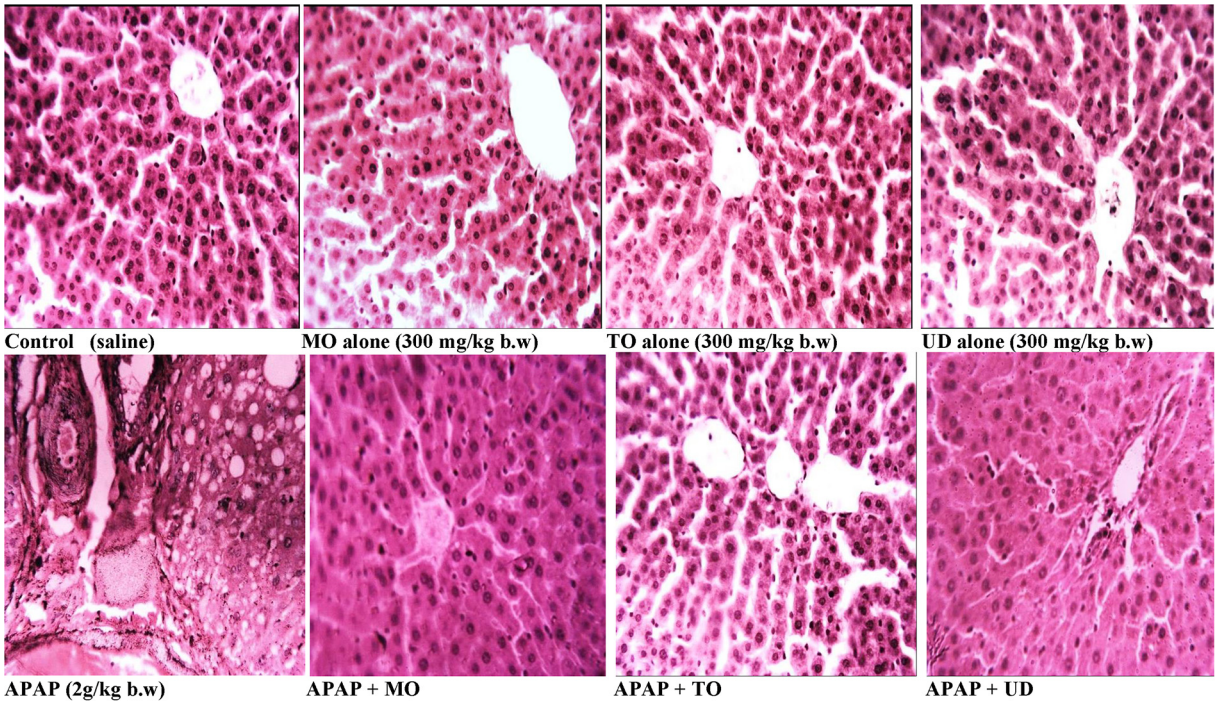

Fig. 5. Histopathology sections from various treatment groups (H&E staining, magnification 40X). Representative section of liver from the control and extracts alone (300 mg/kg b.w oral dose) treated groups showed the normal morphology of tissue. APAP (2 g/kg b.w oral dose) treated rat liver exhibiting greater cellular injuries, loss of hepatic tissue structure organization and assortment of inflammatory cells. Treatment of rats with MO, TO and UD protect liver from APAP induced hepatic injury: Acetaminophen/paracetamol, MO: *Melissa officinalis*, TO*: Taraxacum officinale and* UD*: Urtica dioica.*

Funding

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group no. RGP-193.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to extend their heartfelt thankfulness to the Deanship of Scientific Research at King Saud University, KSA for its support through the research group no. (RGP-193).

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