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Original article

Phytochemical profiling and antioxidant potential of *Daphne mucronata* Royle and action against paracetamol-induced hepatotoxicity and nephrotoxicity in rabbits

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ABSTRACT

The paracetamol-induced injuries of liver and kidneys in animals are mostly used to screen out the hepato and nephroprotective effect of extract or other therapeutic agents. In the present study total phenolic and flavonoid contents, *in vitro* antioxidant, and *in vivo* hepato/nephroprotective (on paracetamol-induced intoxication in experimental rabbits) potentials of the *Daphne mucronata* leaves methanolic extract were determined. For the identification of possible phytochemicals, HPLC (high performance liquid chromatography) analysis was carried out and a total of eight phenolic compounds; malic acid, gallic acid, chlorogenic acid, epigallocatechin gallate, quercetin, morin, ellagic acid, and rutin were identified. *D. mucronata* extract at doses of 250 and 500 mg/kg body weight were given for eight days to paracetamol intoxicated rabbits and the observed results were compared with standard Silymarin. The level of liver enzymes like aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, serum triglyceride, serum cholesterol, serum bilirubin, and kidneys biomarkers like serum urea, uric acid, and creatinine, as well as lipid peroxidation malondialdehyde contents were increased while the antioxidant enzymes like reduced glutathione and total antioxidant capacity were decreased. Furthermore, histopathological analysis of the liver and kidney tissues of control and treated groups also confirmed the hepatoprotective and nephroprotective effect of the *D. mucronata* which was most probably due to its high antioxidant phenolic and flavonoid phytoconstituents.

1. Introduction

In human body, the liver is the major organ that regulates the body homeostasis and control the metabolism of carbohydrates, proteins and fats. In other words liver has a role in almost all body functions in terms of biochemical and metabolic pathways, defence against various diseases, supply of nutrients, involved in reproduction and provision of energy (Rui, 2014; Thorne et al., 2020). In spite of the mentioned functions liver also acts as a storehouse of many valuable biochemical required for different metabolic pathways. In today's world the liver diseases are considered lethal diseases due to causalities caused every year around the globe. In developing countries, the main causes of liver diseases are; hepatitis viruses, environmental poisons, drug, and alcohol

Abbreviations: ABTS, 2, 2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; *D. mucronata*, *Daphne mucronata*; DPPH, 2, 2-Diphenyl,1, picrylhydrazyl; GSH, reduced glutathione; MDA, malondialdehyde; Met. Ext, Methanolic extract; NAPQI, N-acetyl-p-benzo-quineimine; OECD, Organisation for Economic Co-operation and Development; p.o., Per oral; SEM, Standard error mean; TAC, total antioxidant capacity; TFC, Total flavonoid content; TPC, Total phenolic content.

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habits. Some medicines like antibiotics, and other chemotherapeutics (paracetamol etc) also causes hepatotoxicity (Saleem et al., 2010).

Paracetamol, an analgesic and antipyretic drug, is widely used as a hepatotoxic drug in many experimental animals as it causes moderate to severe lobular necrosis. High doses of paracetamol (200 mg/kg and above) is a major cause of liver toxicity and is also a cause of nephrotoxicity (Rehman et al., 2013). Paracetamol overdoses in 10–40% of patients, causes severe liver necrosis and in <2% patients causes renal failure. Its ingestion above therapeutic levels causes acute toxicity in liver, as 90% of it is converted into sulphate and glucuronoid conjugates while the remainder is converted to N-acetyl-p-benzo-quineimine (NAPQI) through cytochrome P450; a highly reactive and toxic intermediate (Kassem et al., 2013). In normal condition it is detoxified through conjugation with hepatic glutathione. Over doses causes the depletion of glutathione (GSH) pool which is an antioxidant enzyme and is involved in detoxification function of the body. Liver and kidney toxicity is mediated by high levels of NAPQI (Jaeschke and Ramachandran, 2020).

The kidneys are also the most important organ of the human body that effectively filters the toxins and waste products from the blood. Kidneys play an important role to regulate our endocrine, acid-base balance, and blood pressure. The renal failure can be acute or chronic depending on exposure to toxins. In chronic state, the kidneys suffer slowly and gradually that last for years that is why renal failure is also referred to as "silent killer". Different free radicals (hydroxyl radicals, hydrogen peroxide, superoxide anions, nitric oxide, nascent oxygen and lipid oxides) produced during metabolic processes are the main causes of liver and kidneys disorders and in cases where glutathione pool is depleted the hazardous effects are more drastic (Levey et al., 2007).

Modern synthetic drugs used to ameliorate hepatotoxicity have minor therapeutic effects and are mostly associated with renal failure. Medicinal plants on the other hands due to its compatibility with human life are more effective in such cases and are associated with no or low side effects (Nazir et al., 2020). Many medicinal plants have shown hepatoprotective and nephroprotective effect in liver and renal lesions induced by paracetamol, gentamicin, profenofos, D galactosamine, chronic stress, cytotoxic drugs, diabetic nephropathy, chemically induced nephrolithiasis by inflammatory mediators and oxidative stress (Vargas-Pozada and Muriel, 2020; Nazir et al., 2020).

The genus Daphne belongs to the family Thymelaeaceae, comprising of almost 90 species, found in Europe, North Africa, and Asia. Mostly these species are used in traditional Chinese and other plant based medication systems for the treatment of various diseases. Experimentally, they have demonstrated a variety of biological and pharmacological effects like antioxidant, antifungal, antibacterial, analgesic, anti-inflammatory, cytotoxic, antiviral, along with abortive and haemostatic effects (Moshiashvili et al., 2020). *D. mucronata* Royle is a wild shrub and is a member of this genus that is widely found in the northern regions of Pakistan and Iran (Katayoun et al., 2003). *D. mucronata* is considered as essential medicinal plant because of its ethnomedicinal and pharmacological uses. It is used as an active ingredient in folkloric medicines to cure various diseases (Rasool et al., 2009). The plant woody bark is recommended to cure bone diseases, relieve eye pain, while the fruits and leaves are used for the treatment of rheumatism (Ghasemi et al., 2012). The extract of the plant has traditionally been used for the treatment of infections related to skin, allergies, and cancer (Malik et al., 2019). Leaves and root extracts of *D. mucronata* have been used in traditional Chinese medicine to treat toothache, ulcers, rheumatism, and as purgative and abortive agent (Katayoun et al., 2003). Its boiled leaves and in ointment form they are used to treat wound infections, constipation, menstrual problems, infertility, and gynaecological complications (Mosaddegh et al., 2012). As mentioned before, extracts from different parts of the Daphne species have a wide range of pharmacological and biological activities which are due to the presence of secondary metabolites in them, suggesting that these plants could serve as a source of active ingredients that could be effectively used in the pharmaceutical, cosmetic, and food industries (Can et al., 2020).

Taking into account the importance of selected plant as depicted by various *in vitro* and *in vivo* studies along with traditional application, the present research study was designed to evaluate the hepatoprotective and nephroprotective effects of the methanolic leaf extract of *D. mucronata* against hepato and nephrotoxicity induced by paracetamol in rabbits. The observed effects were correlated with phenolic and flavonoid contents. In addition HPLC profiling was done to identify possible phenolic and flavonoid compounds where malic acid, gallic acid, chlorogenic acid, epigallocatechin gallate, quercetin, morin, ellagic acid, and rutin were identified through comparison with standards and those reported in literature.

2. Material and methods

2.1. Chemicals and reagents

Antioxidant chemicals such as DPPH, ABTS, ascorbic acid, antioxidant standards, Folin-Ciocalteu regent, and silymarin, were purchased from Sigma-Aldrich (St. Louis, MO, USA); while normal saline solution (Utsoka Pharma, Las Bela Baluchistan, Pakistan), liver profile test kits (Human, Hamburg, Germany), and kidney profile test kits (Biomed: Germany; diagnostic) of the mentioned firms (in brackets) were used in this study. Methanol was purchased from Merck, Darmstadt, Germany.

2.2. Preparation of D. mucronata methanolic extract

The plant leaves were collected from local area and after collection, they were cleaned and kept on a clean paper for 20 days. The shade dried leaves were chopped into small size pieces through mortar and pestle then crushed to fine powder by mechanical grinder. Approximately 8 kg of powder sample was subjected to maceration in 80% methanol for 14 days with periodical shaking. Filtration was carried out through muslin cloth followed by filtration through Whattman filter paper. The filtrates were concentrated into a semisolid mass using rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany) under reduced pressure and then completely dried through lyophiliser (Nazir et al., 2018). The dried mass obtained was 280 g.

2.3. Assessment of total phenolic content

The total phenolic contents (TPC) in the methanolic extract (Met. Ext) were determined using previously reported method (Shirazi et al., 2014). Where Met. Ext (100 μL), distilled water (500 μL), Folin-Ciocalteu reagent (100 μL) and 7% sodium carbonate (1000 μL) were mixed thoroughly and after 90 min absorbance was recorded at 760 nm using UV-Spectrophotometer. TPC in plant samples were measured from the calibration curve of standard Gallic acid and expressed as mg of GAE/gm of dry sample.

2.4. Assessment of total flavonoid contents (TFC)

The TFC were also estimated in *D. mucronata* leaves Met. Ext using previously reported assay (Shirazi et al., 2014). Quercetin

was used as a standard and the enumerated contents were expressed as mg of Quercetin equivalent (mg QE/g) per gram of dry sample of leaves extract. Different dilutions of quercetin were used to draw a calibration curve. Extract (100 μL) mixed with distilled water (500 μL), 5% sodium nitrate (100 μL), 10% aluminium chloride (150 μL) and 1 M sodium hydroxide (200 μL) were mixed thoroughly and after 5 min absorbance at 510 nm was recorded using UV Spectrophotometer.

2.5. Phytochemical profiling of extract by HPLC-UV analysis

For HPLC analysis, about 1 g leaves powder was mixed with water and methanol (20 mL; 1:1 v/v) and then kept in a water bath for 1 h at 70 °C. Finally the sample was cooled and centrifugation was carried out for 10 min at 4000 rpm. The supernatants were then filtered through Whattman filter paper. About 2 mL of it was taken in a properly labelled HPLC vial. Agilent zorbax eclipse (XDB-C18) column was used for the separation of phytochemicals and the resultant chromatogram was compared with that of the reference standards run on the same column (Zeb, 2015). Quantification of phytochemicals was carried out using the following single point calibration formula:

$$\mathrm{Cx} = \frac{\mathrm{Ax} \times \mathrm{Cs}(\mu\mathrm{g/ml}) \times \mathrm{V(ml)}}{\mathrm{As} \times \mathrm{Sample(wt.ing)}} \; Cx = \frac{Ax \times Cs(\mu g/ml) \times V(ml)}{As \times Sample(wt.ing)} \qquad (1)$$

where: *Cx* = sample concentration; *As* = standard peak area; *Ax* = sample peak area; *Cs* = standard concentration (0.09 μg/ml).

2.6. In vitro DPPH (2, 2-diphenyle-1-picrylhyzyl) free radical scavenging potential

DPPH scavenging potential of *D. mucronata* leaves Met. Ext was measured using Brand-Williams method (Brand-Williams et al., 1995). DPPH (0.039 g/100 mL in methanol) solution was prepared and incubated for 30 min in dark. The stock solution of extract (1 mg/mL) was also prepared in methanol from which serial dilutions in the concentration range of 1000, 500, 250, 125, 62.5 and 31.05 μg/mL were then prepared. About 0.1 mL of each working dilution was mixed with 3 mL of DPPH solution and incubated for 30 min at 25 °C. Finally the absorbance of mixture was recorded using UV-spectrophotometer at 517 nm. Same procedure was used for ascorbic acid that was used as standard. %DPPH scavenging potential was measured using the following formula;

$$\%\mathit{Free\ radical\ scavenging\ potential} = \frac{\mathit{Blank\ sample\ absorbance} - \mathit{sample\ absorbance}}{\mathit{Blank\ sample\ absorbance}} \times 100 \qquad (2)$$

2.7. In vitro ABTS (2, 2-azinobis [3-ethylebenzthiazoline]-6-sulfonic acid) scavenging potential

According to the reported procedure (Re et al., 1999), the ABTS free radical scavenging potential was determined as well. ABTS (7 mM) and potassium per sulphate (2.45 mM) solutions were mixed to gather and incubated overnight in dark. About 300 μL of *D. mucronata* Met. Ext working dilutions and 3 mL of ABTS solution were mixed thoroughly and incubated for about 6 min and the absorbance of resulting mixture was recorded via double beam spectrophotometer at 745 nm. Ascorbic acid was used as positive control. The %ABTS free radical scavenging potential of extract was measured using the Eq. (2).

2.8. Animal experiments

The *in vivo* experiments were carried out on forty (40) healthy adults' male rabbits (*Oryctolagus cuniculus*) having initial weight from 1.6 to 2.6 kg. The animals were harboured with 12 h of light–dark cycle at a constant temperature of about 25 ± 3.5 °C and relative humidity of 55 ± 10%, and acclimatized for one week prior to the experiment. The animals were provided free access to water in a well-ventilated room at animal house, University of Malakand, Khyber Pakhtunkhwa, Pakistan. The animals were fed on green vegetables along with fresh grasses. The animals were provided standard food obtainable as *ad libitum* (Nazir et al., 2018). All the animal procedures were conducted according to the ARRIVE guidelines and Animal Scientific Procedure Act; UK (1986) and the approval for the current experimental protocol was taken from the Departmental Animal Ethical Committee (DAEC/2019/1).

2.9. Acute toxicity analysis of D. mucronata Met. Ext

The acute oral toxicity study on the *D. mucronata* leaves Met. Ext was carried out according to the Organization for Economic Co-operation and Development (OECD) guideline 423. Three animals were treated orally with a single dose of 2000 mg/kg body weight of Met. Ext, while other three with distilled water (10 mL/kg) to evaluate the toxic effects if any, in the experimental animals. Immediately after dosing, the rabbits were observed continuously for 2 h for any symptoms of toxicity (convulsions, loss of righting reflex, motor activity, muscle spasm, tremors, lacrimation, sedation, hypnosis, diarrhoea, and salivation). Animals were observed up to 14 days for any signs of toxicity or mortality. The Met. Ext remained safe and nontoxic up to the dose range of 2000 mg/kg body weight. After that the use of an additional upper dose level of $\geq$ 2000 mg/kg body weight (5000 mg/kg, body weight) was tested but no toxic signs were observed in the animals. Therefore, according to the guidelines of OECD, Met. Ext at dose 250 and 500 mg/kg body weight (maximum dose) that was 1/10th of 1500 and 5000 mg/kg dose were given to experimental animals in subsequent experiments to assess the hepatoprotective and nephroprotective effect of the extract (Nazir et al., 2021).

2.10. Animal grouping and dosing

The total study population that was about forty (40) rabbits were grouped into five groups of 8 animals each (Rehman et al., 2015). Each group rabbits were tagged separately for the purpose of identification. Animals of Group I were administered normal saline, p.o., for 8 days, served as normal control group. Group II: Animals were administered with paracetamol 2 g/kg body weight for 8 day. Group III served as standard control group, received paracetamol along with silymarin; a well-known standard hepatoprotective drug (50 mg/ kg p.o) for 8 days. Group IV and V were given *D. mucronata* Met. Ext (250 and 500 mg/kg p.o.) for 8 days along with paracetamol. Immediately after Paracetamol dosing animals were observed for 24 h for signs of toxicity (Rehman et al. 2015). The doses of tested Met. Ext solutions and animals experimental design have been presented in Table 1.

2.11. Hematological and serological profile of the test rabbits

After dosage completion (24 h later on day 9th), all the animals were anaesthetised by injecting anaesthetic combinations (ketamine/xylazine, IM), and euthanized by injecting sodium pentobarbital (100 mg/kg body weight, IV). Onset of sedation was achieved and surgery was performed successfully of all rabbits. Administration of sodium pentobarbital is one of the few

Table 1
Experimental design for *Daphne mucronata* Met. Ext tretament groups used in the study.

Group	Group category	Treatment given	Route/times of administration
I	Normal control	Normal saline (8 mL/kg)	p.o/at 8:00 am and at 8:00 pm (Twice a day)
II	Paracetamol control	Paracetamol (2 g/kg)	p.o/at 8:00 am and at 8:00 pm (Twice a day)
III	Standard control	Paracetamol (2 g/kg b.w) + Silymarin (50 mg/kg b.w)	p.o/at 8:00 am and at 8:00 pm (Twice a day)
IV	Extract treated group	Paracetamol (2 g/kg b.w) + Met. Ext (250 mg/kg)	p.o/at 8:00 am and at 8:00 pm (Twice a day)
V	Extract treated group	Paracetamol (2 g/kg b. w) + Met. Ext (500 mg/kg)	p.o/at 8:00 am and at 8:00 pm (Twice a day)

Met.Ext, Methanolic extract, p.o., Per oral, b.w., body weight.

methods of euthanasia that is listed as acceptable in laboratory rodents. The blood samples (3 mL) were taken by cardiac puncture, transferred to EDTA and non-EDTA containing tubes. The serum was separated by centrifugation for 10 min at 3,000 rpm and 37 °C, stored at 4 °C till determination of biochemical parameters (Cicero et al., 2018).

2.12. Assessment of biochemical parameters

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglyceride, cholesterol, and bilirubin levels in serum were measured using Biochemistry analyser (PS-520; Shenzhen Procan Electronics, China) as per companies' instructions using diagnostics kits (Reactivos, GPL Barcelona, Spain). Kidneys biomarkers like serum urea, uric acid, and creatinine were measured by ROCHE Diagnostic (Reflotron® Plus instrument) kits.

2.13. Assessment of lipid peroxidation and antioxidant biomarkers

The hepatic and renal tissues collected from each rabbit group were excised, cleaned, and immediately perfused with cold saline. The tissues were homogenized in cold phosphate buffer saline (pH 7.4, 0.1 M). Then, the homogenates were filtered and centrifuged (at 3,000 rpm for 20 min). The supernatant was then stored at −80 °C until use for further biochemical analysis of lipid peroxidation and antioxidant biomarkers. Lipid peroxidation was evaluated through measurement of malondialdehyde (MDA) content in the tissues. Oxidative status was assessed by evaluation of the nonenzymatic antioxidant marker; reduced glutathione (GSH) and total antioxidant capacity (TAC) according to the reported assays (Abdel-Daim and Ghazy, 2015).

2.14. Histopathology

For histopathological examination the liver and kidney portions were immediately removed, washed thoroughly with normal saline solution to remove the blood and were processed following the standard protocols (Rehman et al., 2015). The liver and kidney tissues were fixed in formalin (10%), dehydrated with ethanol-xylene mixtures and fixed with paraffin. Tissue blocks were sectioned from 4.5 to 6 μm thickness by Microtome (ACCU-Cut® SRM™; 200 Sakura). Slides were stained with hematoxylin and eosin (H & E) dye using automatic slide stainer (Sakura Tissue-Tek® DRS™ 2000, Japan). The stained slides were cleaned properly and observed under microscope to see alteration in hepatic and kidney tissues architecture of paracetamol intoxicated and treated (Silymarin/extract) groups.

2.15. Statistical analysis

All *in vitro* and *in vivo* experiments were performed in three replicates. All results have been presented as Mean ± SEM. The Student's *t*-test and one way ANOVA followed by Dunnett's *post hoc* multiple comparison test was used to evaluate significance of the data obtained. $P \leq 0.05$ were considered as significant.

3. Results

3.1. Total phenolic/flavonoid content

Results of TPC and TFC of *D. mucronata* leaves extract are presented in Fig. 1. The TPC of *D. mucronata* Met. Ext at various concentrations are presented in Fig. 1A. A calibration curve was drawn for gallic acid (standard) using the dilutions; 1000, 500, 250, 125 and 62.5 μg/mL to assess the TPC while standard quercetin regression curve was made for the estimation of TFC (Fig. 1C) in *D. mucronata* leaves extract.

3.2. Correlation between TPC and TFC versus %DPPH and ABTS inhibition activity

Correlation between TPC and TFC versus antioxidant activities have been presented in Fig. 1 B & D. The highest regression coefficient value (R^2 = 0.9303 and 0.953) was obtained when TPC was plotted against %DPPH and ABTS inhibition (Fig. 1B) while, the correlation coefficient for TFC against % inhibition of DPPH and ABTS were 0.9598 and 0.969 (Fig. 1D) respectively. Regression line for DPPH and ABTS also goes parallel with TPC and TFC indicating a good correlation between the contents and observed inhibition potentials.

3.3. Identification and quantification of possible phytochemicals

A typical HPL-UV chromatogram of *Daphne mucronata* methanolic extract (Met. Ext) is shown in Fig. 2. A total of eight antioxidant phytochemicals were identified. The detailed identification of each antioxidant with their respective peak position in chromatogram and retention time (Rt) are given in Table 2. Malic acid, gallic acid, chlorogenic acid, epigallocatechin gallate, quercetin, morin, ellagic acid, and rutin were eluted at retention time of 2.7, 4.3, 6.0, 8.0, 10.3, 12.0, 16.6, and 22.7 min with the concentration values of 1172.98, 204.91, 70.46, 708.78, 148.04, 45.0, 7.88, and 106.63 μg/ml respectively (Table 2).

3.4. In vitro DPPH free radical scavenging potential

The antioxidant potential of *D. mucronata* Met. Ext displayed a dose dependent response against DPPH free radical. The Met. Ext showed 75 ± 0.55, 71 ± 0.34, 68 ± 0.99, and 63 ± 0.72, and 57 ± 0.79% inhibitions at concentrations of 1000, 500, 250, 125 and 62.5 μg/mL (Table S1 & Fig. 3A) respectively. While standard ascorbic acid caused 90 ± 0.33, 87 ± 0.42, 81 ± 0.36, 77 ± 0.41 and 71 ± 0.75% inhibitions at 1000, 500, 250, 125 and 62.5 μg/mL concentration against DPPH radical respectively.

3.5. In vitro ABTS free radical scavenging potential

The result of %ABTS inhibition potential of D. mucronata leaves Met. Ext are shown in Table S1 & Fig. 3B. The highest observed %inhibition of Met. Ext was 72 ± 0.51 at 1000 μg/mL concentration. Ascorbic acid was used as a standard.

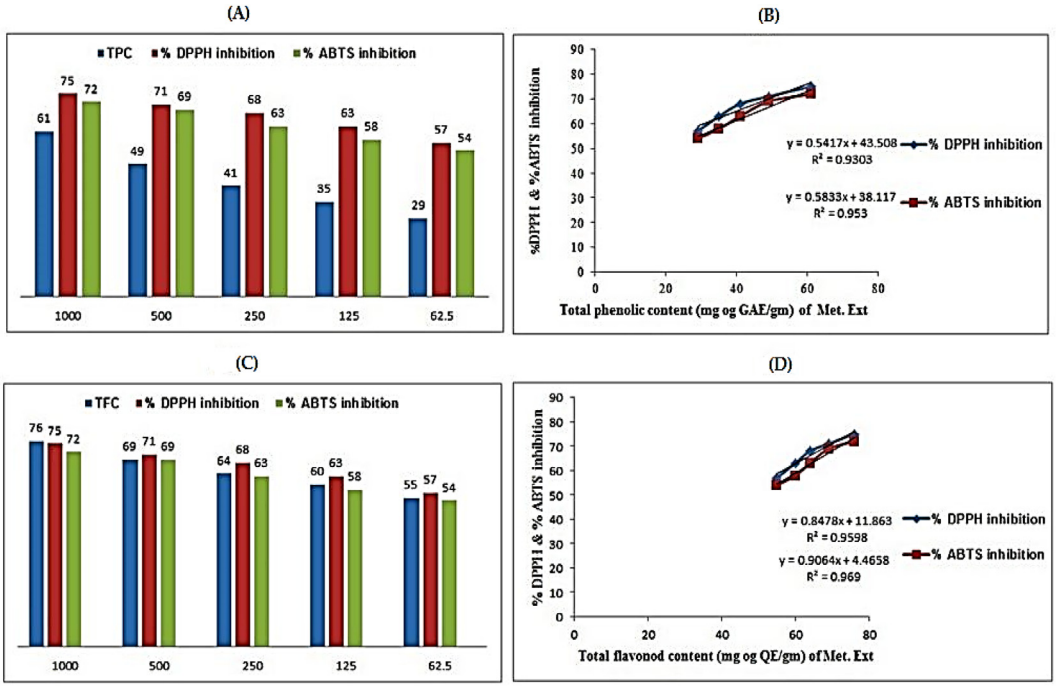

Fig. 1. Total phenolic and flavonoids content of Met. Ext of *Daphne mucronata* and their linear correlation with DPPH and ABTS inhibition potentials. **{(A)** Total Phenolic contents in Met. Ext and free radicals (DPPH & ABTS) inhibition potential at various concentration **(B)** Linear correlation of TPC in Met. Ext vs. % DPPH and %ABTS inhibition **(C)** Total flavonoids content in Met. Ext and free radicals (DPPH & ABTS) inhibition potential at various concentration **(D)** TFC vs. % DPPH and %ABTS inhibition}.

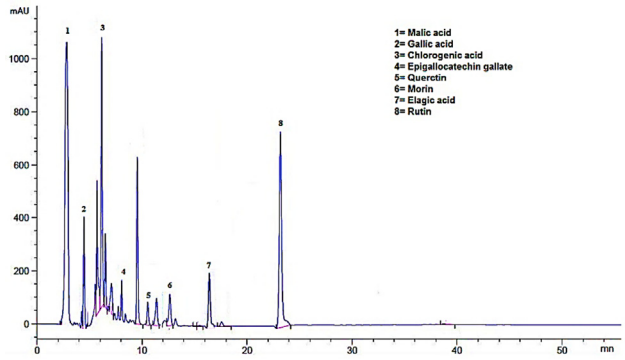

Fig. 2. HPLC chromatogram of *Daphne mucronata* methanolic extract (Met. Ext).

3.6. Hepatoprotective effect of D. mucronata leaves methanolic extract

Hepatotoxicity can be inferred from the increase levels of the biochemical parameter such as serum ALT, AST, ALP, triglyceride, cholesterol and bilirubin. Treatment of rabbits with paracetamol resulted in severe damage to liver which is apparent from high levels of serum ALT, AST, ALP, triglyceride, cholesterol and bilirubin as shown in Table 3 and Fig. 4. Pre-treatment of rabbits with silymarin standard, significantly (***:P < 0.001) reduced the elevated levels of serum ALT, AST, ALP, triglyceride, cholesterol and bilirubin (46 ± 1.5, 82 ± 3.7, 69 ± 1.2, 44 ± 4.0, 34 ± 1.7, 0.5 ± 0.1% respectively) that were induced by paracetamol (Table 3). *D. mucronata* Met. Ext displayed significant (**: p < 0.01, ***: p < 0.001) decrease (36 ± 6.3, 66 ± 6.5, 67 ± 4.7, 50 ± 3.7, 40 ± 4.1, and 0.6 ± 0.2) in all the tested parameters (serum ALT, AST, ALP, triglyceride, cholesterol and bilirubin level, respectively) at 500 mg/kg dose (Fig. 4 A, B, C, D, E, and F) as compared to the paracetamol-treated group. Pronounced dose dependency has been observed for the tested doses of selected plant extract which were comparable to the animal group treated with standard silymarin.

3.7. Nephroprotective effect of D. mucronata leaves methanolic extract

Results of nephroprotective effects of *D. mucronata* Met. Ext on paracetamol intoxicated rabbits are presented in Table 4, and in Fig. 5. A significant increase was observed in kidneys biomarkers like serum urea, uric acid and creatinine (Fig. 5A,B,C) in paracetamol intoxicated animals as compared to control (normal saline) and treated groups (silymarin/extract). Pre-treatment with silymarin (50 mg/kg body weight) reduced the

Table 2
Possible phytochemicals identified in *Daphne mucronata* methanolic extract.

Extract	Peak no	Retention time (min)	Detected phenolic compounds	Sample peak area	Standard peak area	Concentration (μg/ml)
Met. Ext	1	2.7	Malic acid	4727.11	40.32	1172.98
	2	4.3	Gallic acid	4004.04	195.4	204.91
	3	6.0	Chlorogenic acid	90.9	12.9	70.46
	4	8.0	Epigallocatechin gallate	5145.81	72.6	708.78
	5	10.3	Quercetin	1345.72	90.9	148.04
	6	12.0	Morin	9.0	2.02	45.0
	7	16.6	Ellagic acid	251.57	319.24	7.88
	8	22.7	Rutin	238.86	22.4	106.63

Met.Ext, Methanolic extract.

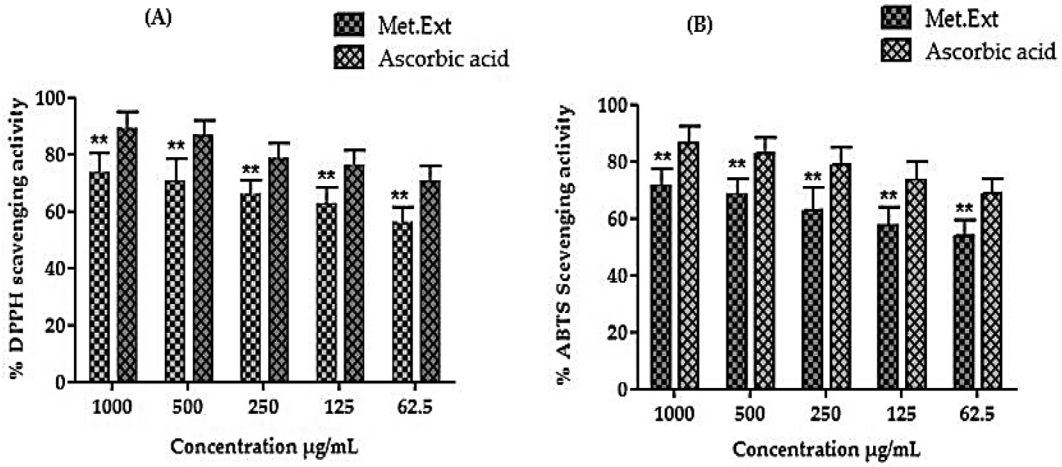

Fig. 3. % Free radical (DPPH and ABTS) scavenging potentials of Met. Ext of *Daphne mucronata* Royle. **{(A)** %DPPH scavenging activity **(B)** %ABTS scavenging activity (** indicates that values were significantly different, p < 0.001)}.

Table 3
Effect of Met. Ext of *Daphne mucronata* on the level of biochemical parameters in paracetamol-induced hepatotoxicity in rabbits.

Groups	Dose	Liver-related parameters with % change values					
		ALT (U/L)	AST (U/L)	ALP(U/L)	Serum Triglyceride (mg/dL)	Serum Cholesterol (mg/dL)	Serum bilirubin (mg/dL)
Normal control	8.0 (mL/kg)	38 ± 2.4***	78 ± 1.2***	66 ± 0.5***	40 ± 0.8***	32 ± 2.0***	0.4 ± 0.2***
Paracetamol control	2.0 (gm/Kg)	94 ± 6.4	124 ± 2.3	185 ± 1.2	75 ± 6.3	68 ± 3.8	1.52 ± 0.3
Silymarin + Paracetamol	50 (mg/Kg)	46 ± 1.5***	82 ± 3.7***	69 ± 1.2***	44 ± 4.0***	34 ± 1.7***	0.5 ± 0.1***
Met. Ext + Paracetamol	250 (mg/Kg)	77 ± 3.2**	71 ± 3.1***	98 ± 6.1***	69 ± 6.1 ns	35 ± 1.2***	0.5 ± 0.1***
	500 (mg/Kg)	36 ± 6.3***	66 ± 6.5***	67 ± 4.7***	50 ± 3.7**	40 ± 4.1***	0.6 ± 0.2***

Each value is presented as mean ± SEM, n = 8 animals. Comparisons have been made between Normal control versus Paracetamol control using student *t*-test (***p < 0.001) and significant differences (*p < 0.05**, p < 0.01***, p < 0.001) were found between the treated groups (Silymarin/Met. Ext) versus Paracetamol control, determined through One way ANOVA followed by Dunnett's posthoc multiple comparison test.

elevated levels of serum urea, uric acid and creatinine. The most effective nephroprotective results were observed for Met. Ext at dose of 500 mg/kg body weight where a significant (**P < 0.01, ***P < 0.001) reduction was observed in serum urea (42 ± 1.2**), uric acid (3.2 ± 4.0***), and creatinine (0.7 ± 0.1 ***) levels.

3.8. Hepatic lipid peroxidation and antioxidant status

Treatment of rabbits with paracetamol significantly ($P \leq 0.001$) increased the hepatic lipid peroxidation marker; MDA level as compared to the normal control group (Fig. 6A). Moreover, paracetamol significantly ($P \leq 0.001$) reduced the liver antioxidant capacity as indicated by declines in GSH (Fig. 6B) and TAC levels (Fig. 6C) compared to the normal control group. Treatment with silymarin (50 mg/Kg) and Met. Ext (250 & 500 mg/kg) significantly (**$P \leq 0.01$; ***$P \leq 0.001$) reinstated the antioxidant capacity in the rabbit liver homogenate to the normal level, that restored the GSH and TAC levels comparable to that of the control group values. In the silymarin (50 mg/Kg) and Met. Ext (250 & 500 mg/kg) treated groups, the MDA level was significantly ($P \leq 0.001$) decreased, while that of the antioxidant markers like GSH and TAC were significantly (**$P \leq 0.01$; ***$P \leq 0.001$) high in comparison with the paracetamol treated group. These results indicates the synergistic hepatoprotective and antioxidant effects of *D. mucronata* leaf extract (Fig. 6).

3.9. Renal lipid peroxidation and antioxidant status

The effects of paracetamol, silymarin (50 mg/kg), and Met. Ext (250 & 500 mg/kg) supplementation on the renal lipid peroxidation and antioxidant activities are demonstrated in Fig. 6. Parallel to results of hepatic MDA and oxidative status, paracetamol intoxication significantly (***$P \leq 0.001$) elevated the renal MDA level (Fig. 6A) and reduced GSH (Fig. 6B) and TAC levels (Fig. 6C) as compared to the normal control group. In the same manner, daily administration of silymarin (50 mg/Kg) and Met. Ext at the dose of 250 mg/kg and 500 mg/kg, significantly (***$P \leq 0.001$) restored the antioxidant activities to the normal levels in liver. Silymarin and Met. Ext (250 & 500 mg/kg) significantly (***$P \leq 0.001$) reduced MDA level and increased GSH and TAC levels significantly ($P \leq 0.01$; $P \leq 0.001$) as compared to the paracetamol treated group.

3.10. Histopathological evaluation of the liver tissues

Histopathological slides of the animals liver tissues treated with *D. mucronata* leaves Met. Ext and paracetamol are presented in Fig. 7. Animals of Group I (control group) shows normal liver parenchyma and the endothelia linings of central veins with normal morphology and no evidence of necrosis. The liver sections in group II animals treated with paracetamol, reveals necrosis, inflammation, bile duct proliferation, and cholestasis. Group III liver histopathology slides shows that the animals treated with standard drug silymarin shows a normal liver architecture. Histopathology of group IV animals, treated with Met. Ext (250 mg/kg) shows a mild bile duct ingestion and scattered inflammatory cells. Improved and normal histological architecture were seen in animals of group V treated with Met. Ext (500 mg/kg).

3.11. Histopathological evaluation of the kidney tissues

Protective effects of *D. mucronata* leaves Met. Ext on paracetamol induced nephrotoxicity are presented in Fig. 8. Histopatholog-

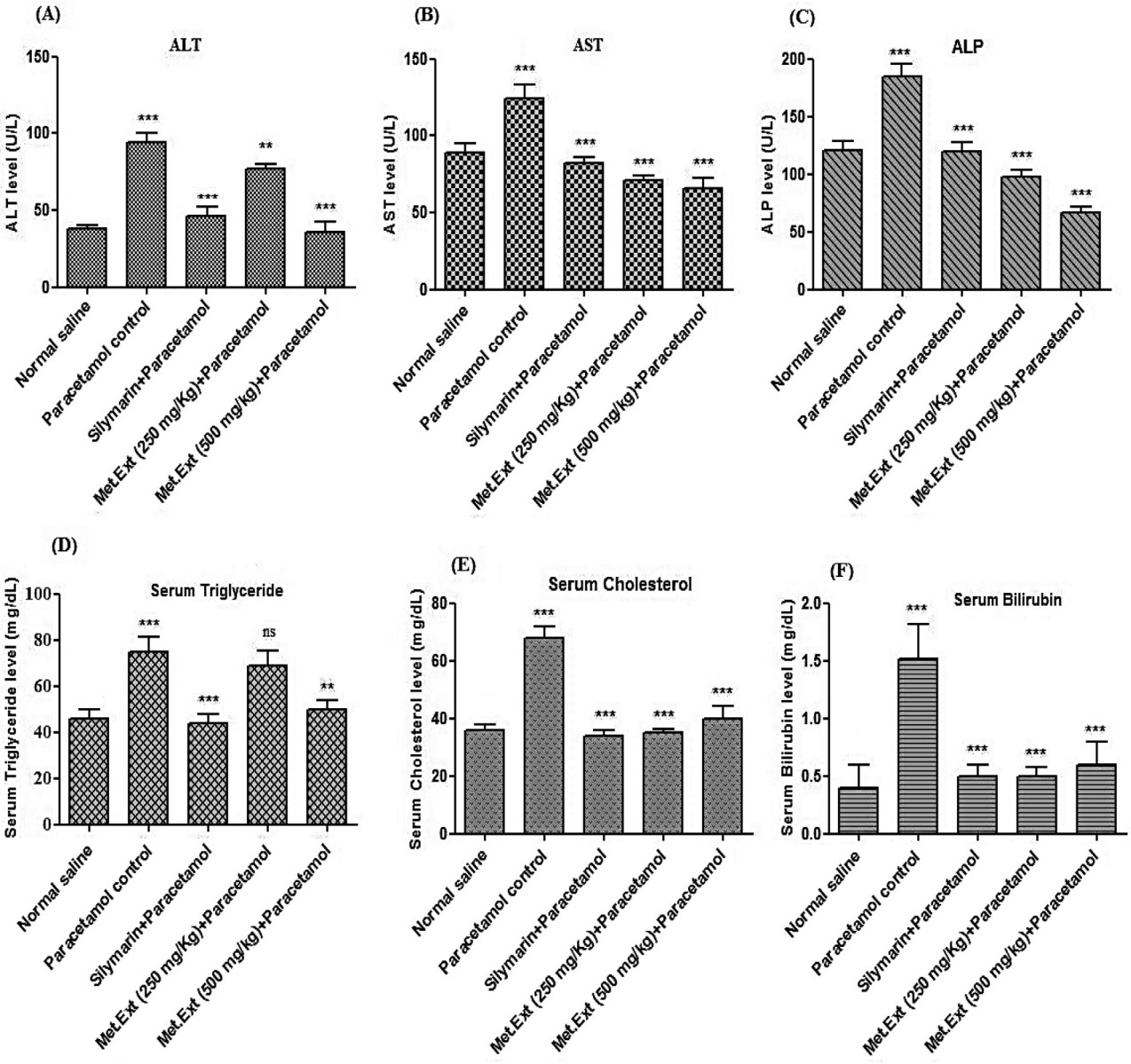

Fig. 4. Effects of Met. Ext of *Daphne mucronata* on the level of *liver* serum biochemical parameters in paracetamol-induced hepatotoxicity in rabbits **{(A)** serum level of ALT, **(B)** serum level of AST, **(C)** serum level of ALP, **(D)** serum level of Triglyceride, **(E)** serum level of cholesterol **(F)**, and serum level of bilirubin in paracetamol-intoxicated rabbits model: Values are presented as mean ± SEM, n = 8. Significant difference (***p < 0.001) between the normal control versus paracetamol control have been determined using student *t*-test and **p < 0.01 and ***p < 0.001 indicates that values are significantly different from that of the treated groups (Silymarin/Met. Ext) in comparison to paracetamol control that have been estimated through One way ANOVA followed by Dunnett's posthoc test}.

Table 4
Effects of Met. Ext of *Daphne mucronata* on the level of kidney serum biochemical parameters in paracetamol-induced hepatotoxicity in rabbits.

Groups	Dose	Kidney related parameters with % change values		
		Serum urea (mg/dL)	Serum uric acid (mg/dL)	Serum creatinine (mg/dL)
Normal control	8.0 (mL/kg)	37 ± 0.5***	3.2 ± 1.1***	0.4 ± 0.1***
Paracetamol control	2.0 (gm/Kg)	92 ± 2.3	8.2 ± 6.3	2.5 ± 0.3
Silymarin + Paracetamol	50 (mg/Kg)	39 ± 3.8***	3.7 ± 3.7***	0.6 ± 0.1***
Met. Ext + Paracetamol	250 (mg/Kg)	61 ± 4.4*	4.8 ± 6.3**	0.8 ± 0.2***
	500 (mg/Kg)	42 ± 1.2**	3.2 ± 4.0***	0.7 ± 0.1 ***

Each value has been presented as mean ± SEM, n = 8 animals. Comparisons have been made between normal control versus paracetamol control groups using student *t*-test (***p < 0.001) while significant differences (* p < 0.05,** p < 0.01, ***p < 0.001) were found between the treated groups (Silymarin/Met. Ext) in comparison to paracetamol control group determined through One way ANOVA followed by Dunnett's posthoc multiple comparison test.

ical slide of Rabbit's kidney of Group I (normal saline) shows normal glomeruli and flat epithelium lining glomerular capsule with distinct capsular space. Animals of Group II, treated with paracetamol shows somewhat necrosis and inflammation. Histopathology

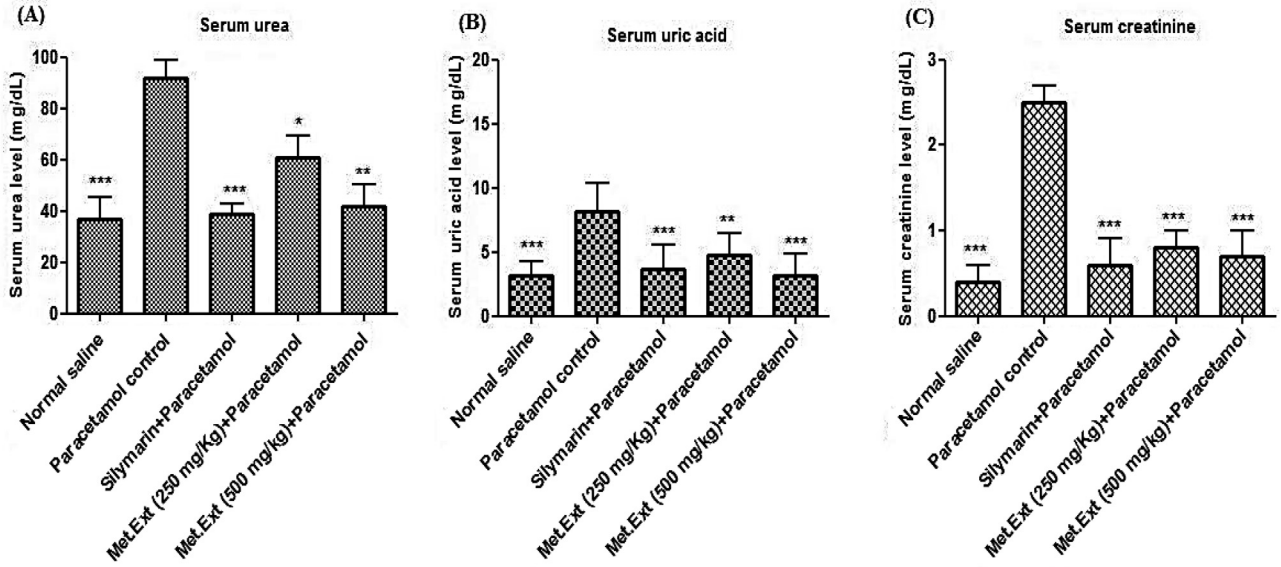

Fig. 5. Effects of Met. Ext of *Daphne mucronata* on the level of kidney serum biochemical parameters in paracetamol-induced nephrotoxicity in rabbits. **{(A)** serum level of urea **(B)**, serum level of uric acid, and **(C)**, serum level of creatinine in paracetamol-intoxicated rabbits model: Values are presented as mean ± SEM, n = 8. Significant difference (***$p < 0.001$) between the normal control group versus paracetamol control group have been observed that were determined using student *t*-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicates that values are significant and have been determined for treated groups (Silymarin/Met. Ext) versus paracetamol control group using One way ANOVA followed by Dunnett's posthoc multiple comparison test}.

of kidney from Group III animals treated with standard silymarin shows normal parenchyma with no significant changes. Group IV animals, treated with Met. Ext. (250 mg/kg) shows normal renal parenchymal tubules with the normal lumen, while for Group V rabbits treated with Met. Ext (500 mg/kg) normal renal parenchyma with no significant pathology were observed.

4. Discussion

In the current study, hepatotoxicity is evident from the high levels of biochemical parameters such as ALT, AST, ALP, serum triglycerides, cholesterol and bilirubin in paracetamol treated groups as compared to the standard silymarin group. The paracetamol treatment increases the level of the mentioned liver biomarkers that results in excessive damage to liver cells. The plant extracts reinstated/restored the damage caused by paracetamol which has been observed in animal models for different plant extracts. Similar results have been observed in this study as well. Previous studies on *D. mucronata* phytochemical analysis have shown that it contains coumarins, flavonoids, triterpenoids, diterpene, sterols, lignin cumarinolignans, glucosides, daphnecin, aquillochin, daphnine and umbelliferone (Al-Snafi et al., 2019). Other biologically active compounds isolated from this plant previously includes; 5, 7, 3′, 4′-tetrahydroxyflavone and 5, 3′, 4′-trihydroxyflavone 7-O-β-D-glycopyranoside that have exhibited antioxidant potentials as well (Rasool et al., 2009). The plants phenols, diterpenes, and coumarins are associated with significant antioxidant activities (Shah et al., 2018). There is a close relationship between antioxidant activities and phenolic compounds (Yener et al., 2020). In the current study HPLC profiling of *D. mucronata* leaf extract showed the presence of phenolic and flavonoid compounds like malic acid, gallic acid, chlorogenic acid, epigallocatechin gallate, quercetin, morin, ellagic acid, and rutin. Among them, chlorogenic acid, epigallocatechin gallate, quercetin, and ellagic acid have previously exhibited strong antioxidant and hepatoprotective activities (Zhang et al., 2014; Afifi et al., 2018; Ashraf et al., 2018; Aljelehawy et al., 2020). Plants have antioxidant properties depending on the content of phenol and flavonoids, due to which they may play a role in scavenging free radicals thus exhibiting protective effects in oxidative stress and related complications in animal models (Nazir et al., 2021).

Since paracetamol toxicity demonstrates itself through oxidation pathways, ROS and MDA levels (as oxidative markers), GSH and TAC levels (as antioxidant indices) are evaluated in liver and kidney tissues is such situations. In the current study, an increase in lipid peroxidation have caused a significant increase in MDA level and decrease in the levels of antioxidants such as GSH and TAC that indicates the oxidative stress, which is in agreement with the other reported studies in literature (Sohrabinezhad et al., 2019; Ashfaq et al., 2020). Studies have shown that the enzymatic and non-enzymatic systems that maintain cellular homeostasis, as well as the level of lipid peroxidation, are markedly influenced by paracetamol (Belinskaia et al., 2020). The severity of the damage in terms hepatotoxicity and nephrotoxicity induced by paracetamol was evident from an increase in the serum levels of the lipid peroxidation indicator; malondialdehyde and decreased levels of GSH and TAC (which serves as indices of antioxidant status). Significant decreases in GSH and TAC levels have also been observed in the liver, kidney, and blood of patients with liver disease compared to controls in a reported study (Afsharinasab et al., 2020). In recent years, a large number of plants have been tested to eradicate the liver damage caused by paracetamol in animal models, and the bioactive compounds responsible for alleviating oxidative stress have been isolated (Meharie et al., 2020). The predominant and responsible metabolite of paracetamol; NAPQI that is responsible for liver damage, in high concentration causes a decrease in GSH and TAC levels in hepatocytes. Overproduction of NAPQI causes a break down of the SH group present in macromolecules such as proteins, nucleic acids, and membranes leading to liver damage. Under reduced GSH level, excessive NAPQI covalently bound to vital proteins sandwitched between lipid bilayer in membranes of hepatocytes, resulting in lipid peroxidation and increased MDA levels. The improvement in GSH levels in the liver alleviates both the initiation and the progression of liver damage caused by paracetamol (Jaeschke et al., 2020).

The kidney controls plasma levels of ions like sodium, potassium, calcium, magnesium, and chloride and are importantly

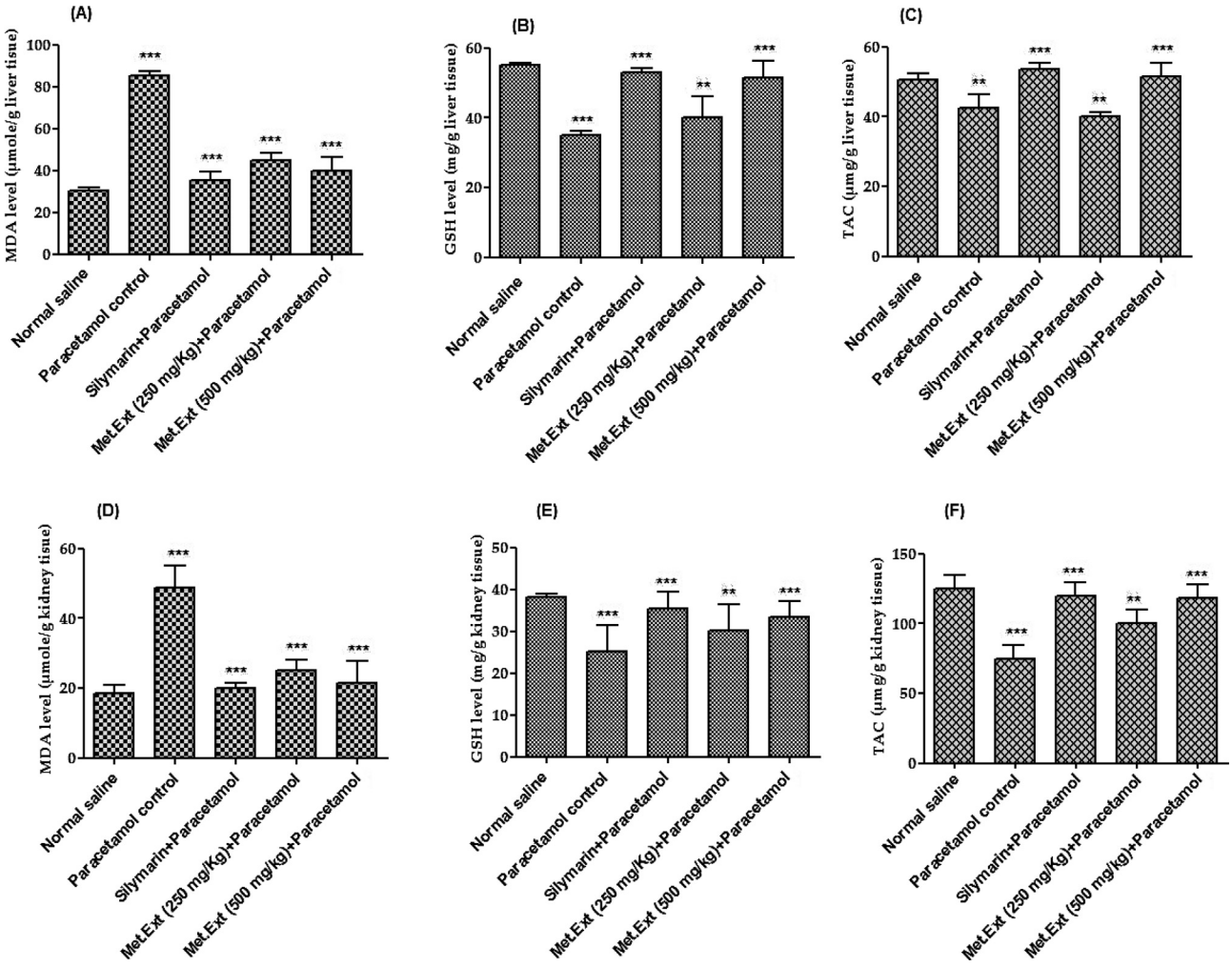

Fig. 6. Effects of *Daphne mucronata* leaf Met. Ext and silymarin on liver and kidney lipid peroxidation and antioxidant biomarkers in paracetamol-induced toxicity in rabbits. {Lipid peroxidation and antioxidant biomarkers in liver: **(A)** MDA level **(B)** GSH level **(C)** TAC level. Lipid peroxidation and antioxidant biomarkers in kidney: **(D)** MDA level **(E)** GSH level **(F)** TAC level. Values are presented as mean ± SEM, n = 8. Significant differences (***p < 0.001) between the normal control and paracetamol treated groups were observed which were estimated using student *t*-test. **p < 0.01 and ***p < 0.001 indicated that values were significantly different for the treated groups (silymarin/Met. Ext) in comparison to paracetamol control group determined through One way ANOVA followed by Dunnett's posthoc multiple comparison test}.

involved in the removal of nitrogenous waste products such as urea, uric acid, and creatinine from the body. Increase in the levels of kidney parameters like serum urea, uric acid and creatinine are usually monitored to assess the drug induced nephrotoxicity in man and also in animal models (Aziz, 2019). Our results are in agreement with the reported studies where pre-treatment with silymarin (50 mg/kg) standard significantly decline the elevated level of serum creatinine, urea and plasma ions induced by Paracetamol (Kassem at al., 2013).

The observed effects in terms of biochemical parameters were further supported by histopathological examination. Histological architecture of the liver sections of paracetamol intoxicated rabbits displayed a significant necrosis, inflammation, bile duct proliferation, and cholestasis. Paracetamol treated rabbits showed cuboidal epithelial cell necrosis in proximal convoluted tubules which are also in line with the reported studies (Rehman et al., 2015). The histopathological examination revealed that *D. mucronata* extract protect liver and kidneys of intoxicated animals. The standard silymarin (A standard hepatoprotective drug) greatly reduced the cellular impairment caused by paracetamol due to its strong antioxidant potential (Nazir et al., 2018), and thus reduces the hepatotoxicity and lipid peroxidation caused by a variety of other agents as well. In the current study the protective effects of *D. mucronata* Met. Ext were comparable to that of the standard silymarin.

Medicinal plants and their metabolites are of great importance in the present modern world and are in use from centuries. However, before use as therapeutic agent, it is needed to assess their pharmacological and toxicological effects scientifically (Jothy et al., 2012). Keeping in view the importance of medicinal plant; *D. mucronata,* the current study was carried out to assess the hepatoprotective and nephroprotective effect intoxicated rabbits. Literature studies revealed that plants contain valuable phytoconstituents which are responsible for the observed hepatoprotective and nephroprotective activities (Akomolafe et al., 2014; Domitrović et al., 2014; Nehal et al., 2018; Ghosia et al., 2018; Bhatia et al., 2019; Kuzu et al., 2019; Prasad and Prasad, 2019). The results of this study suggests that possible mechanism of this protection might be due the scavenging capabilities of phytoconstituents (mostly phenolics) present that have effectively scavenged the free radicals produced in intoxicated animals. However further studies are required to evaluate the exact mechanism of action.

To the best of our knowledge, there was no reported hepatoprotective and nephroprotective studies on *D. mucronata*. Therefore, this study was designed to report the contents of phenolic acids

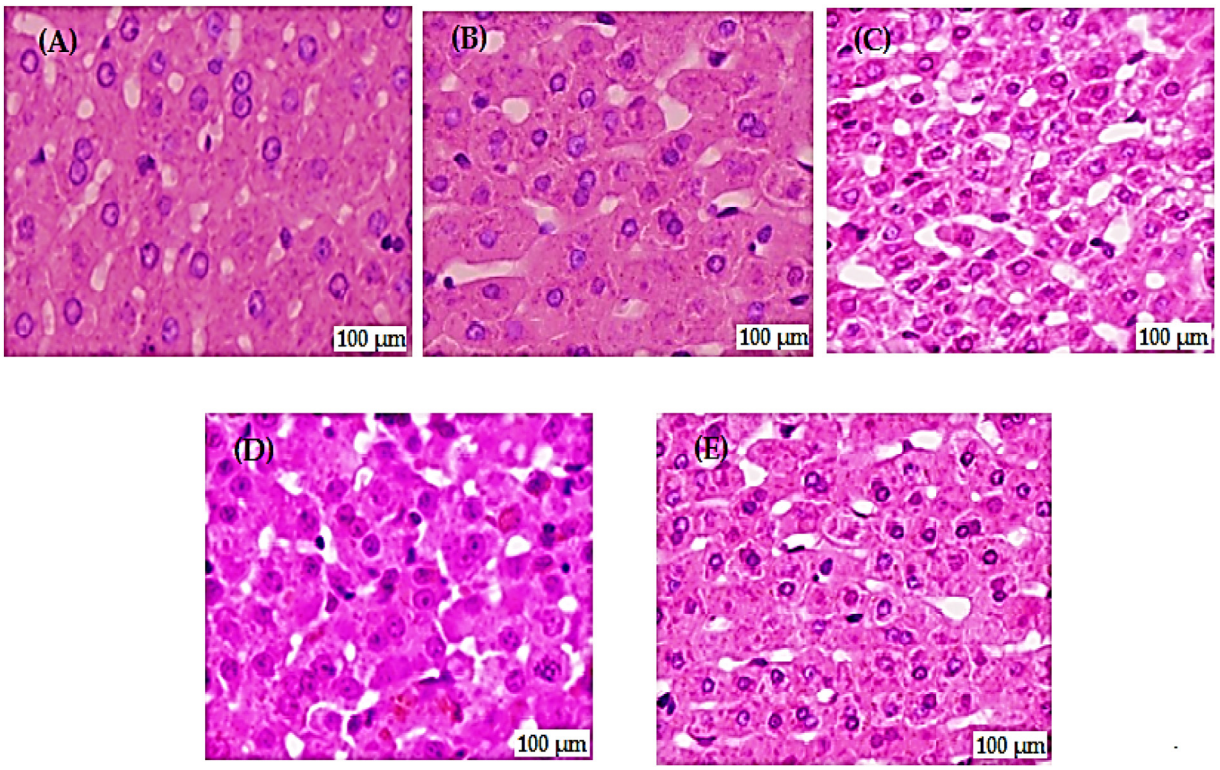

Fig. 7. Effects of Met. Ext of *Daphne mucronata* on histopathology studies in paracetamol-induced hepatotoxicity in rabbits. **{(A)** Liver of control group displaying normal liver parenchyma and the endothelia linings of central veins had normal morphology with no evidence of necrosis, **(B)** Section of liver parenchyma from a rabbit of Group II treated with paracetamol reveals necrosis, inflammation, and bile duct proliferation, **(C)** Histopathology of a liver from Group III treated with standard Silymarin shows almost recovery of the normal architecture with normal hepatocytes arrangement, **(D)** Histopathology of a liver from Group IV treated with Met.Ext (250 mg/kg) exhibiting with mild bile duct ingestion, scattered inflammatory cells are seen, **(E)** Histopathology of a Liver from Group V of rabbits treated with Met. Ext (500 mg/kg) shows normal histological appearance and Improved architecture is observed in groups; (H & E staining; 40X & 100X, scale bar = 100 µm)}.

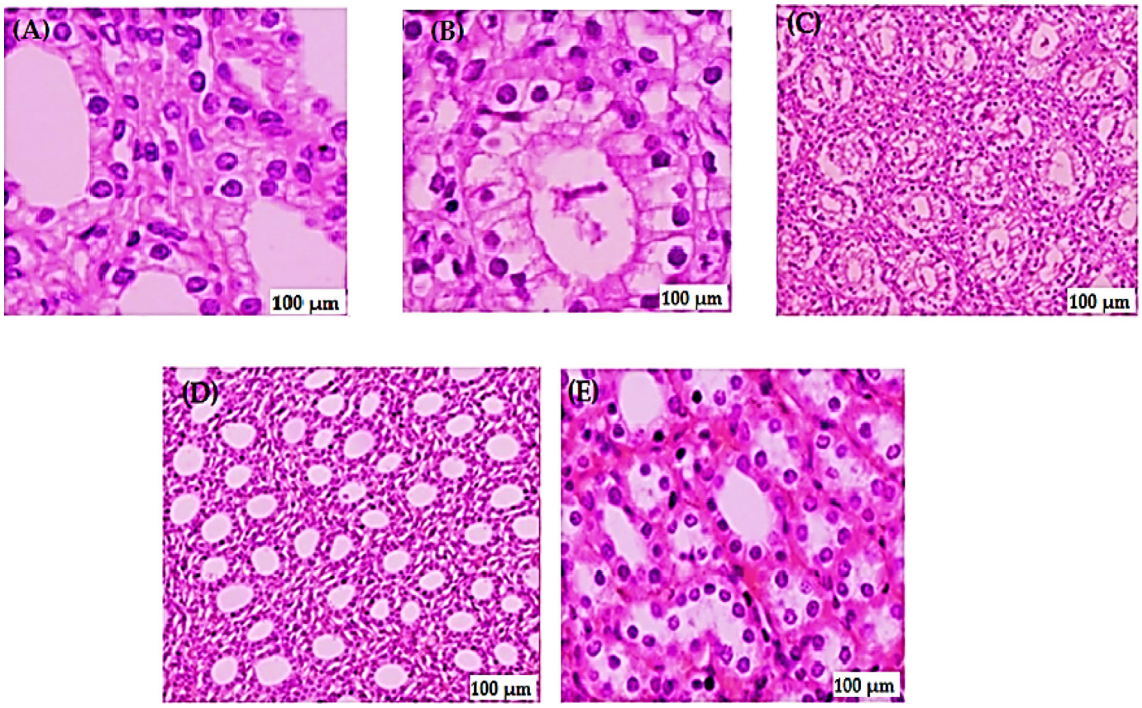

Fig. 8. Effects of Met. Ext of *Daphne mucronata* on histopathology studies in paracetamol-induced nephrotoxicity in rabbits. **{(A)** Histopathological slide of Rabbit's kidney of Group I showing normal glomeruli and flat epithelium lining glomerular capsule with distinct capsular space, **(B)** Histopathological slide of Rabbit's kidney of Group II, treated with paracetamol shows somewhat necrosis and inflammation, **(C)** Histopathology of a kidney from Group III treated with standard Silymarin shows normal parenchyma with no significant changes, **(D)** Histopathology of a kidney from Group IV, treated with Met. Ext. (250 mg/kg) shows normal renal parenchymal tubules with the normal lumen, **(E)** Improved architecture is observed in groups of rabbits treated with Met. Ext (500 mg/kg) indicating normal renal parenchyma with no significant pathology; (H &E staining; 40X & 100X, scale bar = 100 µm)}.

and flavonoids which most probably were responsible *in vitro* antioxidant activity and *in vivo* hepatoprotective and nephroprotective effects. *D. mucronata* leaves Met. Ext ameliorated paracetamol intoxicated acute liver and kidney injuries in rabbits, evidenced by the outcomes of both biochemical and histological studies.

5. Conclusion

The results of the current study revealed that leaves extract of *D. mucronata* have *in vitro* antioxidant and *in vivo* hepatoprotective, and nephroprotective potentials and is capable of decreasing elevated levels of liver biochemical parameters like AST, ALT, ALP, serum triglyceride, serum cholesterol, serum bilirubin, and kidneys biomarkers like serum urea, serum uric acid, and serum creatinine levels in paracetamol intoxicated rabbits. *D. mucronata* extract also inhibited the physiological and histological changes in liver and kidney in addition to decreasing effect on lipid peroxidation MDA content. The extract has also increased the level of antioxidant enzymes like GSH and TAC, and therefore, it can be inferred that the extract might be capable of preventing hepatic and renal toxicity through enhancing hepatic and kidney tissue oxidant/antioxidant balance. These effects might be due to the presence of phenolic and flavonoids phytoconstituents present in the methanolic extract of *D. mucronata.* Anti-oxidative therapy, especially the use of natural antioxidants, is an affordable therapeutic approach for the prevention of oxidative stress related diseases of liver and kidney. Keeping in view the high medicinal importance of *D. mucronata* it could be recommended for reducing hepatic impairments and related diseases of liver and kidney and could also be used as bioceutical or dietary supplement in controlling liver and kidney diseases. However, further experiments are needed to assess its permissible doses and toxicities in other animal models along with isolation of responsible compounds.

Declarations

Ethics approval

All procedures related to the animal activities have been approved by the Departmental Animal Ethical Committee of the University of Malakand (DAEC/2019/1) and were conducted according to the ARRIVE guidelines and UK: Animal Scientific Procedure Act (1986). These guidelines were in accordance with the internationally documented principles for laboratory used and care.

Consent for publication

Not applicable for this submission.

Availability of data and materials

The data presented in this manuscript belong to the research work done by Mr. Jabran Muhammad under the supervision of Dr. Nausheen Nazir and has not been deposited in any repository yet. However, the materials are available to the researchers upon request.

Authors' contributions

JM & NN carried out plants collection and experimental work. NN carried out literature search and manuscript preparation and supervised the research project. NN, RG & JM carried out *in vivo* experimental work. Statistical analysis was done by FU. RU and AA carried out formal analysis. Corrections and editing of the manuscript was done by NN, AA, MZ, and MN. MZ revised the paper. All the authors have read and finally approved the manuscript.

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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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.05.051.

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