Phytochemical analysis and comprehensive evaluation of pharmacological potential of *Artemisia brevifolia* Wall. ex DC

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**Abstract**

Multitude of diseases and side effects from conventional drugs have surged the use of herbal remedies. Thus, the current study aimed to appraise various pharmacological attributes of *Artemisia brevifolia* Wall. ex DC. Extracts prepared by successive solvent extraction were subjected to phytochemical and multimode antioxidant assays. Various polyphenolics and artemisinin derivatives were detected and quantified using RP-HPLC analysis. Compounds present in methanol (M) and distilled water (DW) extracts were identified using high resolution mass spectrometry (HRMS). Extracts were pharmacologically evaluated for their antibacterial, antifungal, antimalarial, antileishmanial and antidiabetic potentials. Moreover, cytotoxicity against *Artemiasalina*, human cancer cell lines and isolated lymphocytes was assessed. Genotoxicity was evaluated using comet, micronucleus and chromosomal aberration assays. Lastly, anti-inflammatory potential was determined through a series of *in vitro* and *in vivo* assays using BALB/c mice. Maximum extract recovery (5.95% w/w) was obtained by DW extract. Highest phenolics and flavonoids content, total antioxidant capacity, total reduction potential, percent free radical scavenging, β-carotene scavenging and iron chelating activities were exhibited by M extract. RP-HPLC analysis revealed significant amounts of various polyphenolic compounds (vanillic acid, syringic acid, emodin and luteolin), artemisinin, dihydro artemisinin, artesunate and artemether in ethyl acetate (EA) extract. Total 40 compounds were detected through HRMS. A noteworthy antimicrobial activity (MIC 22.22 μg/ml) was exhibited by EA extract against *A. fumigatus* and several bacterial strains. Maximum antimalarial, antileishmanial, brine shrimp lethality and cytotoxic potential against cancer cells was manifested by EA extract. None of the extracts exhibited genotoxicity and toxicity against isolated lymphocytes. Highest α-amylase and α-glucosidase inhibition capacities were demonstrated by DW extract. Various *in vivo* anti-inflammatory models revealed significant (p < 0.05) anti-inflammatory pharmacological potential of *A. brevifolia* and endorse its richness in artemisinin.

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**Abbreviations:** CL, Comet Length; DMEM, Dulbecco Modified Eagle’s Medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DMSO, Dimethyl sulfoxide; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; HRMS, High Resolution Mass Spectroscopy; IC50, 50% Inhibitory Concentration; LD50, Lethal Dose Causing 50% Mortality; MIC, Minimum Inhibitory Concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MN, Micronucleus; NAMP, Number of Aberrant Metaphase; NDI, Nuclear Division Index; RP-HPLC, Reverse Phase-High Performance Liquid Chromatography; ZOI, Zone of Inhibition.

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1. Introduction

Plants are an integral part of life on earth. They form the basis of traditional medicinal systems since ancient times. An incredibly growing tendency to use natural remedies has been witnessed both in developed and developing countries. This is quite evident from the fact that more than 40% of prescribed drugs in the world are mainly derived from herbal sources. The process of natural product drug discovery involves multifaceted scientific approaches including ethnopharmacology and reverse pharmacology. These trans-disciplinary endeavors extend from gathering the folk knowledge of indigenous people to quasi drug development and provision of better and safer lead compounds (Shelar and Shirote, 2011). Legacy of traditional medicines established over hundreds of years by hit and trial on human subjects comprehend the valuable biomedical information, which can be uncovered through modern approaches. One such underexplored folklore is Artemisia brevifolia Wall. ex DC. (tarkha), from genus Artemisia, which is one of the largest and most widely distributed genera of the Asteraceae family. Several of its species are frequently utilized for the treatment of malaria, hepatitis, cancer, inflammation and microbial infections. Genus Artemisia (Wormwood, Mug wood, Sagebrush or Tarragon) is rich in aromatic plants, which by virtue of the presence of monoterpene and sesquiterpenes possess a characteristic scent or taste. These compounds also are responsible for their application in folk medicine (Koul et al., 2018).

A. brevifolia, a woody rooted herb, is the native plant of Northern India, Pakistan and Afghanistan. Growing widely in stony landscapes of Pakistan, A. brevifolia abounds in remote areas of Baltistan, Chitrál, Gilgit, Swat, Khaghan, Deosai plains and Astor. The understudy species grows in stony topography with low humidity, sandy soils and usually covers the sun facing inclines of dry mountains. A. brevifolia is a 14–35 cm tall herb with a characteristic aromatic fragrance and whitish green color. Its stems have a purplish shade. Flowering period of the plant is from August to September (Ashraf et al., 2010).

Traditionally, its decoction and powder are used as purgative and vermifuge. It is also employed to treat a number of ear, nose and throat related ailments as well as respiratory and gastric disorders by different native communities of Pakistan (Nadeem et al., 2013). Very limited scientific data is available regarding its pharmacological uses and only its anthelmintic use has been validated (Ashraf et al., 2010). A. brevifolia, though abundantly growing, is considered a troublesome weed and its dried stems are used as a source of fire (Hayat et al., 2009).

There is no evidence of systematic and organized bio-chemical screening of this species that could demonstrate the pharmacological potential of this ethnomedicinally important plant. Thus, the present study was planned to transform a bio-waste into a therapeutically active drug candidate by employing a battery of bioassays. Chemical and pharmacological profile of the plant was established by analyzing its extracts of escalating polarity for the presence of phytochemical compounds, antioxidant, antimicrobial, cytotoxic, antiprotozoal and enzyme inhibition potential. To the best of our knowledge, it is the first report highlighting pharmacological potential of A. brevifolia.

2. Material and methods

2.1. Chemicals and solvents

Solvents (n-Hexane, ethyl acetate, methanol, dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), lamda carrageenan, sea salt, naphthylendiamine, croton oil, gallic acid, artemisinin, artesunate, artemether, dihydroartemisinin, syringic acid, luteolin, tween 80, linoleic acid, gentisic acid, human serum and histopaque-1077 were bought from Sigma-Aldrich (Germany). Folin-Ciocalteu (FC) reagent, amphotericin B, clotrimazole, cefixime, roxithromycin and phosphate buffer (PB) were purchased from Riedel-de-Haen (Germany). Sabourad dextrose agar (SDA), α-glucosidase enzyme, α-Amylase enzyme, EDTA, RPMI-1640 culture media, Dulbecco’s modified eagle medium (DMEM), sodium bicarbonate (NaHCO3), normal melting point agarose (NMPA), low melting point agarose (LMPA) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder, N-2-hydroxyethylpiperezine-N-2-ethanesulfonic acid (HEPES), trishHCl, sodium carbonate (Na2CO3), starch, p-nitrophenyl-D-glucopyranose, sulphanalidase, phosphoric acid, acetonitrile, emodin, vanillic acid, β-carotene, folic acid, catechin, hydroethidine, trypsin, cytochalasin B, gimsa stain and colchicine were bought from Merck, KGaA (Darmstadt, Germany). Acetic acid, triton X-100, ethidium bromide, dried instant yeast, heat inactivated fetal bovine serum (HIFBS) and acarbose were bought from Merck-Schuchardt (USA). Unless otherwise stated, all chemicals were procured from Sigma-Aldrich (USA).

2.2. Cell lines

Human cancer cell lines including leukemia cell line (THP-1, ATCC-TIB-202), hepatocellular carcinoma cell line (Hep G2, ATCC-HB-8065), ovarian cancer cell line (A2780, ATCC-CRL-2772), hormone responsive breast cancer cell line (MCF-7, ATCC-HTB-22), colorectal adenocarcinoma cell line (HT-29, ATCC-HTB-38) and prostate cancer cell line (DU-145, ATCC-HTB-81) were used in the study.

2.3. Microbial strains

Nine bacterial strains including Klebsiella pneumoniae (ATCC-1705), Staphylococcus aureus (ATCC-6538), Bacillus subtilis (ATCC-6633), Escherichia coli (ATCC-25922), Pseudomonas aeruginosa (ATCC-15442), Resistant Pseudomonas aeruginosaa (MIC-103), Methicillin Resistant Staphylococcus aureus (MIC-104), Resistant Escherichia coli (MIC-102) and Resistant Streptococcus hemolyticus (-MIC-101) were tested in this study. Fungal strains including Aspergillus flavus (FCBP-0064), Muor species (FCBP-0300), Aspergillus niger (FCBP-0198), Fusarium solani (FCBP-0291) and Aspergillus fumigatus (FCBP-06) were used. Chloroquine-sensitive Plasmadium falciiparum D6 strain (Sierra Leone PD6), chloroquine-resistant P. falciiparum W2 strain (Indochina PFW2) and Leishmania tropica (W23 were utilized.

2.4. Artemia salina eggs

Brine shrimp eggs, acquired from Ocean star International, USA has been utilized.

2.5. Animals

About 7 weeks old Balb/c mice, weighing ~25–30 g of either sex were used in the study. All animals were housed under standard 12-h light and dark cycles with food and water provided ad libitum. Animals were acclimatized to the laboratory environment for 30 min before the experiment.

2.6. Ethical statement

After taking, written informed consent, blood was collected from two healthy female individuals (less than 30 years) by
venipuncture to isolate lymphocytes. Donors confided that they were not recently exposed to drug treatment, smoking, radiation or any viral infection. International ethical guidelines were followed after approval by the Ethical Board of the Quaid-i-Azam University, Islamabad, Pakistan (Letter # BEC-FBS-QAU-042/2019). Guidelines for the care and use of laboratory animals narrated by National Institute of Health, USA were adhered with.

2.5.2. Detection of artemisinin and its derivatives
Artemisinin and its analogs were quantified using previously defined protocols with minor modifications (Fatima et al., 2015). Each extract (10 mg/ml) was dissolved separately in HPLC grade methanol. All the solutions weresonicated, centrifuged and filtered through 0.2 μm sartolon polyamide membrane filter. Artemisinin standards (artesunate, artethermethyl, dihydroartemisinin and artesminin) were prepared in HPLC grade methanol with the final concentration of 250 ppm and were eluted at flow rate of 1 ml per min with injection volume of 50μlμl using Agilent Chem station Rev. B.02-01-SR1 (260) and Agilent 1200 series binary gradient pump coupled with diode array detector (DAD). Reverse phase chromatographic analysis was carried out with a Zorbex-C8 analytical column (4.6 × 250 mm, 5 μm particle size), mobile phase A (acetonitrile) and mobile phase B (phosphate buffer) at gradient method of 0–3 min for 0–70% B, 3–7 min for 70–60% B, 7–10 min for 60–50% B and then 70% B until 30 min. The absorption of samples was recorded at 210 nm.

2.8.2. Total flavonoid content
Previously described aluminum chloride colorimetric method with slight modification was adopted (Ahmed et al., 2017). Briefly, 20 μl of each extract (4 mg/ml DMSO) was added to respective wells of 96 well plate. This reaction mixture was incubated for 30 min and read at 415 nm with the help microplate reader. Results were calculated in microgram quercetin equivalent per milligram of the extract (μg QE/mgE) at different concentrations of quercetin (40, 20, 10, 5, 2.5 and 0 μg/ml). Assay was run in triplicate.

2.8.3. RP-HPLC analysis
(1) Detection of artemisinin and its derivatives
RP-HPLC profiling was done for quantitative analysis of polyphenols using 8 standards by previously described procedure (Ahmed et al., 2017). HPLC grade methanol was used to extract the samples and re-filtered using 0.45 μm membrane filters (Millex-HV). The HPLC system was Agilent Chem station Rev. B.02-01-SR1 (260) and Agilent 1200 series binary gradient pump coupled with diode array detector (DAD). Analytical column Zorbex-C8 (4.6 × 250 mm, 5 μm particle size) with 50 μl of injection volume was used. Mobile phase consisted of acetonitrile-methanol–water-acetic acid in a ratio of 5:10:85:1 (solvent A) and acetonitrile-methanol-acetic acid in a ratio of 40:60:1 (solvent B). Gradient method was 0–20 min for 0–50% B, 20–25 min for 50–100% B and then isotonic 100% B until 30 min. Flow rate was maintained at 1 ml/min. The column was reconditioned for 10 min every time before next analysis. Polyphenols were identified by comparing retention time and UV–Vis spectra of chromatographic peaks with reference standards at 257 nm (vanillic acid) and 279 nm (gallic acid, syringic acid, emodin), 325 nm (luteolin, ferulic acid, gentisic acid).

2.9. Biological evaluation

2.9.1. Antioxidant potential of A. Brevifolia extracts
(1) Percent free radical scavenging activity (FRSA)
FRSA of the extracts was evaluated using methanol solution of DPPH (Ahmed et al., 2017, Baig et al., 2020). An aliquot of 10 μl of the extracts (4 mg/ml DMSO) were added to respective wells of 96 well plate. DPPH solution (190 μl) was then added in each
well followed by incubation for 1 h at 37°C in dark. Absorbance was noted at 517 nm using microplate reader. Ascorbic acid was used as positive control whereas DMSO was used as negative control. Percent FRSA was calculated according to the given formula;

\[
\text{Percent FRSA} = \left[ 1 - \left( \frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}} \right) \right] \times 100
\]

Extracts having FRSA ≥ 50% were further examined at lower concentrations of 200, 66.6, 22.22 and 7.4 μg/ml to evaluate their 50% inhibitory concentration (IC\textsubscript{50}) in μg/ml. The assay was performed in triplicate.

(2) **Total antioxidant capacity**

Phosphomolybdenum based total antioxidant capacity was determined using ascorbic acid as positive control (Nasir et al., 2020). Briefly, 100 μl of each extract (4 mg/ml DMSO) was mixed with 900 μl of TAC reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate solution in water) in Eppendorf tubes followed by incubation in water bath at the temperature of 95°C for 90 min. Tubes were cooled to room temperature from which 200 μl of each sample was transferred to 96 well plate and absorbance was measured at 630 nm using microplate reader. The assay was done in triplicate. Antioxidant potential was measured in microgram ascorbic acid equivalent per milligram of the extract (μg AAE/mgE). DMSO was used as negative control.

(3) **Total reducing power**

Reducing power of the extracts was evaluated using standard potassium ferricyanide colorimetric assay (Fatima et al., 2015). Briefly, 200 μl of extract samples (4 mg/ml DMSO) was mixed with 400 μl each of phosphate buffer (0.2 M, pH 6.6) and 1% (w/v) potassium ferricyanide followed by an incubation at 50°C for 20 min. After the addition of 200 μl of 10% (w/v) TCA, Eppendorf tubes were centrifuged at 3000 rpm for 10 min. About 150 μl of supernatant was transferred to respective wells of 96 well plate followed by the addition of 50 μl of 0.1% (w/v) FeCl\textsubscript{3}. Absorbance was measured at 630 nm. Ascorbic acid served as positive control at 6.25–100 μg/ml final concentrations. DMSO was used as negative control. Assay was performed in triplicate. Results were expressed as μg AAE/mgE.

(4) **β-carotene scavenging activity**

Capability of extracts to inhibit the β-carotene bleaching was recorded (Majid et al., 2015). Reaction mixture was prepared by dissolving β-carotene (2 mg/ml) tween 80 (200 mg) and linoleic acid (20 mg) in 10 ml of chloroform. Afterwards, a uniform emulsion of β-carotene linoleate was prepared by evaporating the chloroform using vacuum followed by the addition of distilled water (50 ml). An aliquot of 30 μl of extract was mixed with 250 μl of β-carotene linoleate emulsion. Zero h reading was taken at 470 nm. Reaction mixture was kept at 45°C for 2 h and then final optical density was recorded. Catechin was used as positive standard whereas DMSO infused disc was used as a negative control. Assay was performed in triplicate. Percent of inhibition of β-carotene bleaching was calculated using the underlying formula:

\[
\text{Percent of inhibition} = \left[ 1 - \left( \frac{A_a(120) - A_c(120)}{A_c(0) - A_a(120)} \right) \right] \times 100
\]

where A\textsubscript{a}(1 2 0) and A\textsubscript{c}(1 2 0) are the absorbance of antioxidant and control, respectively taken after 2 h whereas A\textsubscript{a}(0) and A\textsubscript{c}(0) are the zero h readings of antioxidant and control.

(5) **Iron chelating activity**

Procedure described by Majid et al. (2015) was followed. About 200 μl of extract sample, 100 μl of iron (II) chloride dihydrate (2.0 mM) and 900 μl of methanol were mixed carefully followed by an incubation period of 5 min. Reaction mixture was again incubated at 37°C for 10 min after the addition of 400 μl of ferrozine (5.0 mM). Spectrophotometry was performed at 562 nm. EDTA was used as positive standard. The assay was done in triplicate. Underlying formula was used to calculate the percent of chelating effect;

\[
\text{Percent of chelating effect} = \left( \frac{Ac - As}{Ac} \right) \times 100
\]

where Ac and As are the absorbance of control and samples respectively.

2.9.2. **Antimicrobial potential of A. brevifolia extracts**

(1) **Antibacterial assay**

Previously described disc diffusion method with slight modifications was utilized to examine the antibacterial potential of extracts against nine bacterial strains (Zahra et al., 2017). An aliquot of 100 μl of inoculum with pre-adjusted seeding density was swabbed slowly in three different directions over the surface of nutrient agar media plates. Sterile filter paper discs loaded with 5 μl (20 mg/ml DMSO) of extracts were placed on the surface of the plates. Roxithromycin and cefixime loaded discs were utilized as positive control whereas DMSO infused disc was used as a negative control. After an incubation of 24 h at 37°C, zone of inhibition (ZOI) was measured using Vernier calliper. Minimum inhibitory concentration (MIC) was determined for the extracts showing zones greater than 12 mm by standard three-fold microbroth dilution method. Lowest concentration of the extract, which depicted a visible ZOI was considered as MIC. The assay was done in triplicate.

\[
\text{Percent of growth inhibition} = \left[ 1 - \left( \frac{\text{Turbidity of Test Sample}}{\text{Turbidity of Negative Control}} \right) \right] \times 100
\]

Lowest concentration exhibiting ≥90% bacterial growth inhibition was declared as MIC. The assay was done in triplicate.

(2) **Antifungal assay**

Antifungal potential of extracts was assessed by disc diffusion method against five fungal strains (Nasir et al., 2017). Fungal spores were harvested in 0.02% Tween 20 solution. McFarland 0.5 turbidity standard was utilized to adjust turbidity. About 100 μl of fungal spore suspension was swabbed on SDA plates followed by the placement of the extract loaded sterile filter paper discs (5 μl; 20 mg/ml DMSO). Clotrimazole and DMSO impregnated disc were used as a positive and negative controls, respectively. Plates were then incubated for 24–48 h at 28°C. Diameter of ZOI around each disc was measured using Vernier calliper. Extracts producing ZOI ≥ 10 mm were screened to determine MIC at lower concentrations ranging from 200 to 7.4 μg/disc by standard disc diffusion method. Lowest concentration of the extract, which depicted a visible ZOI was considered as MIC.
2.9.3. Antiprotozoal potential of A. brevifolia extracts

(1) Antileishmanial activity

Previously described MTT assay with slight modifications was employed to evaluate the in vitro antileishmanial potential of extracts (Waseem et al., 2017). A 6–7 days incubated L. tropica promastigotes culture was used. Parasites were cultivated in RPMI 1640 Medium containing 100 IU/ml benzyl penicillin, 100 µg/ml streptomycin sulphate and 10% fetal bovine serum (PBS) at 24°C. About 180 µl of parasitic culture at a seeding density of 1 × 10⁶ promastigotes/ml was transferred to respective wells of 96 well plate already containing 20 µl (100 µg/ml) of extracts (prepared in ≤1% DMSO in PBS). Amphotericin B (0.33 to 0.004 µg/ml) was used as positive control. DMSO (1%) in PBS was used as negative control. After incubation for 72 h at 24°C, 20 µl of sterilized MTT solution (4 mg/ml in distilled water) was added to each well and plates were incubated for 4 h at 24°C. Afterwards, supernatant was discarded carefully in order not to disturb the colored water insoluble formazan crystals, which were dissolved in 100 µl of DMSO and stabilized for 60 min. Absorbance was recorded at 540 nm using microplate reader. Following formula was used to determine percent of cytotoxicity:

\[
\text{Percent of cytotoxicity} = 100 - \left( \frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100 \right)
\]

Samples exhibiting >50% cell mortality were further analyzed at lower concentrations, i.e., 33.3, 11.1, 3.7 and 1.23 µg/ml. Table curve 2D v5.01 software was used to calculate IC₅₀.

(2) Antimalarial activity

Antimalarial activity of the thee extracts was evaluated against the chloroquine sensitive and resistant strains of P.falciparum, i.e., D6 and W2 according to previously defined methodology (Kauw et al., 2008). Human erythrocytes were cultured in A+ blood and suspended in RPMI 1640 medium supplemented with HEPES (25 mM), 10% human serum and NaHCO₃ (25 mM). Strains were subjected to incubation in a humidified atmosphere of 5% CO₂ at 37°C. Parasitemia was maintained between 1 and 5% with non-parasitized blood for adjustment of instrument. Assay was executed in triplicate in 96 well plate containing 20 µl of respective extracts and 180 µl of parasite culture at 2% hematocrit and parasitemia. Extracts were tested at a concentration of 100 µg/ml. Chloroquine was used as positive whereas 1% DMSO was used as negative control. Plates were incubated for 48 h in a CO₂ atmosphere at 37°C. Briefly 190 µl of harvested culture with seeding density of 1 × 10⁷ cells/ml was added to respective wells of 96 well plate followed by the addition of 10 µl of the A. brevifolia extracts (1% DMSO in PBS) with a final concentration of 20 µg/ml. cisplatin, 5-flourouracil, vincristine and doxorubicin (4 mg/ml DMSO) were used as positive control. DMSO (1%) in PBS was used as negative control. After an incubation at 37°C for 24 h, MTT assay was performed as described in Section (2.9.3.1). Extracts exhibiting >50% activity were further screened at lower concentrations, i.e., 10, 5, 2.5 and 1.25 µg/ml. Assay was repeated thrice. IC₅₀ was calculated using table curve 2D v5.01 software.

(3) Cytotoxicity against isolated lymphocytes

Previously described protocol with slight modifications was used for lymphocytes isolation (Waseem et al., 2017). About 3 ml of blood was collected from healthy volunteer after venipuncture and diluted (1:1) with PBS. After layering over 2 ml of Histopaque-1077, blood was centrifuged at 800g for 20 min. Buffy coat, formed in the center, was aspirated in 5 ml of PBS and centrifuged for 4 min at 350 rpm. Growth medium, RPMI-1640 (1 ml) was used to suspend the pellet and cell density was adjusted to get 1 × 10⁵ cells/ml. For cytotoxicity determination, 20 µl of extracts at the final concentration of 20 µg/ml was incubated with 180 µl of lymphocyte with a seeding density of 1 × 10⁵ cells/ml, at 37°C for 24 h in humidified 5% CO₂ incubator. Vincristine (4 mg/ml) or 1% DMSO in PBS were used as positive and negative controls, respectively. Afterwards, previously described MTT assay (2.9.3.1) was performed.

2.9.5. Genotoxicity assays

(1) Comet assay

Comet assay method with slight modifications was employed for genotoxicity evaluation of the extracts (Kazmi et al., 2018). Briefly, cancer cell lines were cultured in DMEM medium (with low glucose and 4 mM L-glutamine) supplemented with 10% v/v HIFBS, 100 U/ml benzyl penicillin and 100 µg/ml streptomycin sulphate. About 180 µl of monolayer respective cell lines cultures were seeded at a density of 5 × 10⁴ cells/well and allowed to adhere overnight under an atmosphere of 5% CO₂ at 37°C. After an incubation of 24 h, cells were treated with 20 µl of the extracts with a concentration of 100 µg/ml and incubated again for 72 h.

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under the same conditions. Cisplatin at the final concentration of 0.5 μg/ml was used as positive control. Later on, the cells were trypsinized using a mixture of EDTA and trypsin and centrifuged at 100g, for 5 min at a temperature of 4°C in 150 μl of molten 0.5% LMPA. The cell suspensions were quickly spread on microscopic slides pre-coated with 85 μl of 1% NMPA and covered with cover-slip (25 mm × 25 mm). Agarose was solidified by keeping the slides at 4°C for 5 min. Coverslips were detached carefully and another layer of 75 μl LMPA was added. Slides were exposed to lysis solution (2.5 M NaCl, 0.1 M Na2-EDTA, 0.01 M trishCl, 1% Triton X-100 and 10% DMSO) for a period of 2 h at 4°C. Later, slides were kept in an electrophoresis tank containing alkaline electrophoresis buffer (pH 13) and allowed to sit in for 20 min for unwinding the DNA. Slides were then electrophoresed for a period of 20 min (300 mA; 25 V). Tris buffer (pH 7.5) was used to neutralize the alkali. Slides were stained with 80 μl 1X ethidium bromide and left for 7 min. Chilled distilled water was used to remove the excess stain. Coverslip was then placed and slides were examined immediately using fluorescent microscope. Images were scored using CASP 1.2.3.b software. For each sample, nearly 50–100 cells were observed for comet length (CL), head length (HL), percent of DNA in head as well as tail, tail length (TL) and tail moment (TM) of nuclei for each sample.

(2) Micronucleus assay

Micronucleus assay with cytokinesis block was conducted according to previously described procedure (Tsuboy et al., 2007). Cancer cell lines were cultivated as monolayer cultures in DMEM media supplemented with 10% HIFBS, 100 U/ml benzyl penicillin and 100 μg/ml streptomycin sulphate and incubated under humidified atmosphere of 5% CO2 at 37°C. Cell suspensions at a density of 2.5 × 106 cells were grown in 25 cm² flasks. After 48 h, observing a confluence of 60–70%, medium was removed and cells were treated with extracts at a concentration of 100 μg/ml. For antimutagenicity testing, the cultures were simultaneously exposed to the combination of cisplatin and extracts or cisplatin alone (at the final concentration of 0.5 μg/ml). After 24 h of incubation, cells were again incubated with media supplemented with cytochalasin B having a final concentration of 6 μg/ml for a period of 24 h. Later on, cells were exposed to cold hypotonic solution of KCl (5.6 g/l) and centrifuged at 800 rpm for 8 min. Cell were fixed using acetic acid:methanol (1:3) and 2 drops of formaldehyde. Slides were coded and stained using 5% aqueous Giemsa solution for a period of 15 min. About 1000 binucleated cells (BN) per culture with intact cytoplasm were scored for the incidence of percent micronucleation formation (%MN). Nuclear division index (NDI) was calculated for each experimental point.

(3) Chromosomal aberration assay

Chromosomal aberration assay was performed according to previously described protocol to detect the potential antiallgenic and antialastogenic effects of A. brevifolia using cancer cell lines with a seeding density of 3.8 × 106 cells/dish (Imreova et al., 2017). When a confluence of 70% was observed, seeded cells were incubated with the A. brevifolia extracts at a concentration of 100 μg/ml for 24 h. Later on old medium was replaced with fresh medium while positive control dishes were treated with the media containing mutagen cisplatin (0.5 μg/ml). Cells were cultivated for 42 h. An intact control group was mounted without extract. Colchicine at a concentration of 0.75 μg/ml was added 3 h before the end of incubation. Cells were counted before karyological processing. Slides were stained using 10% aqueous Giemsa solution for 10 min. Microscopic examination was executed to evaluate the chromosomal aberrations. About 100 metaphases were analyzed for each sample. Structural aberrations including chromatid, iso-chromatid, breaks and exchanges were observed.

2.9.6. In vitro anti-inflammatory activity

Nitric oxide scavenging potential of extracts was assessed following the protocol narrated by Majid et al. (2015) with minor modification. Equal volumes of 0.1% naphthenedianide (1 mg/ml) in distilled water and 1% of sulphanilamide (10 mg/ml) in 5% phosphoric acid were added to prepare Griess reagent. About 100 μl of both, samples and sodium nitroprusside (10 mM), were mixed followed by the addition of 1 ml of the Griess reagent and incubation at room temperature for 3 h. Absorbance was read at 546 nm using ascorbic acid as a positive control. Following formula was used for determining the percent of nitric oxide radical formation inhibition.

\[
\text{Percent of inhibition} = \left( \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \right) \times 100
\]

2.9.7. In vivo anti-inflammatory activity

(1) Experimental design for in vivo anti-inflammatory assays

About 7 weeks old Balb/c mice (n = 5) weighing ~25–30 g of either sex were used in the study. Animals were housed in aluminum cages under hygienic conditions (12 h light/dark cycle, 25 ± 1°C temperature) at primate Facility of Quaid-i-Azam University Islamabad, Pakistan. All the animals were supplied with standard chow feed along with water ad libitum prior to use in experiments. Mice were randomly divided into 11 groups, where group I-III were designated as Normal, Vehicle and Positive control groups which received normal saline only, 10% DMSO in 0.75% carboxymethyl cellulose solution and ibuprofen (10 mg/kg), respectively. Group IV-XI were treatment groups, which were designated as NHL, NHN, EAL, EAH, ML, MH, DWL and DWH, where L and H represents low (150 mg/kg body weight) and high dose (300 mg/kg body weight), respectively. Dose was administered orally. Carrageenan suspension (0.9% w/v in normal saline) was used to induce hind paw edema whereas 0.2 ml of 6% croton oil in diethyl ether was used to induce anal edema.

(2) In vivo carrageenan induced paw edema inhibition assay

Anti-inflammatory prospects of the extracts were determined with the help of Carrageenan-induced hind paw edema model described by Majid et al.(2018) with slight modifications. Extracts were administered 60 min before injecting 150 μl of carrageenan suspension in planter aponeurosis. Hind paw edema volume (mm³) was immediately recorded by measuring paw circumference after injection (zero h) and was noted again after time interval of 1, 2, 3 and 4 h using Plethysmometer (Ugo Basile 7140, Italy). Results were calculated as:

\[
\text{Percent of inhibition} = \left( \frac{\text{Control Animal Edema Vol} - \text{Test Animal Edema Vol}}{\text{Control Animal Edema Vol}} \right) \times 100
\]

(3) In vivo croton oil induced anal edema inhibition

Previously described noninvasive protocol was used to assess the croton oil induced edema response on anus of test animals (Majid et al., 2018). Cotton swab soaked with inducer was rubbed for 10 sec on anus of mice around 60 min after the oral administra-
tion of specified doses. Readings were noted for 1st, 2nd, 3rd and 4th h using Vernier calliper. Anti-inflammatory potential was determined by reduction in anal edema by comparing it with positive control.

\[ \text{Percent of inhibition} = \frac{(\text{Control Animal Edema Vol} - \text{Test Animal Edema Vol})}{\text{Control Animal Edema Vol}} \times 100 \]

### 2.9.9. Enzyme inhibition assays

1. \( \alpha \)-amylase inhibition assay

   Antidiabetic potential of the extracts was determined through \( \alpha \)-amylase inhibition assay following standard protocol with slight modification (Ahmed et al., 2019). Reaction mixture containing 15 \( \mu \)l of PBS (pH 6.8), 25 \( \mu \)l of \( \alpha \)-amylase enzyme (0.14 U/ml) followed by the addition of 10 \( \mu \)l extract (4 mg/ml DMSO) and 40 \( \mu \)l of starch (2 mg/ml in potassium phosphate buffer) was incubated for 30 min at 50 °C. Aliquot of 20 \( \mu \)l of 1 M HCl was then added to respective wells of 96 well plate to stop the reaction. Finally, 90 \( \mu \)l of iodine reagent (5 mM iodine, 5 mM potassium iodide) was added. Acarbose solution and DMSO were added to the positive and negative control wells instead of extracts whereas blank was added. Absorbance of Control Animal Edema Vol – Test Animal Edema Vol / Control Animal Edema Vol \times 100

2. \( \alpha \)-glucosidase inhibition assay

   Previously described procedure was employed for evaluating the \( \alpha \)-glucosidase inhibitory potential of the samples (Ovais et al., 2018). An aliquot of 30 \( \mu \)l of \( \alpha \)-glucosidase glucopyranoside (substrate) solution was added into respective wells of 96 well plate along with 69 \( \mu \)l of 50 mM phosphate buffer (pH 6.8). Later, 5 \( \mu \)l of each extract (4 mg/ml DMSO) and 1 \( \mu \)l of enzyme (3 units/ml in 50 mM phosphate buffer; pH 6.8) were added. Initial reading at 405 nm was taken immediately using microplate reader. IC\(_{50}\) was determined for the samples. Percent of inhibition was calculated with the help of given formula:

\[ \text{Percent of inhibition} = \frac{(\text{Absorbance of Sample} - \text{Absorbance of Negative Control}) \times 100}{(\text{Absorbance of Blank} - \text{Absorbance of Negative Control})} \]

### 2.10. Statistical analysis

Data obtained in this study was presented as mean ± SD. The results obtained for phytochemical and cytotoxic assays were analyzed statistically using computer software Statistix 8.1. Significant differences among the results obtained were calculated by Tukey’s multiple comparisons. Statistical significance was set at \( p < 0.05 \). Graphs were generated using software GraphPad Prism 5.

### 3. Results

#### 3.1. Percent extract recovery

Percent recovery of extracts prepared using solvents of successive polarity has been summarized in the Table 1. Highest extraction recovery was observed by DW extract (5.95 w/w) followed by M (4.25% w/w) and EA (2.75% w/w) extracts. The lowest percent yield (1.5% w/w) was obtained in NH solvent.

#### 3.2. Phytochemical analysis

1. **Total phenolic content estimation**

   Total phenolic content of the extracts were calculated by calibration curve \( y = 0.0943x-0.0661, R^2 = 0.9906 \) (Table 4). M extract depicted the maximum (\( p < 0.05 \)) phenolic content, followed by DW and EA extracts, respectively. Minimum phenolic content was found in NH extract. Phenolic content decreased in extracts in following trend: M > DW > EA > NH.

2. **Total flavonoid content estimation**

   Total flavonoid content are expressed in \( \mu \)g QE/mgEand calculated by calibration curve \( y = 0.0649x-0.0403, R^2 = 0.9927 \) (Table 4). Highest flavonoids (\( p < 0.05 \)) were found in M extract followed by DW and EA extracts, respectively. Lowest flavonoids were quantified in NH extract.

   **RP-HPLC analysis**

   1. Detection of artemisinin and its derivatives

      RP-HPLC analysis of A. brevifolia extracts was conducted to detect and quantify artemisinin and analogues (Table 2, Fig. 1). EA extract depicted maximum quantities of artemisinin (50.7 ± 0.15 mg/mgE) and artemether (81.4 ± 0.3 mg/mgE), artesunate (155 ± 0.20 mg/mgE) and artemether (81.4 ± 0.3 mg/mgE). All these sesquiterpenes were found in NH extract as well, with maximum quantity of DH artemisinin, i.e., 643 ± 0.19 mg/mgE. M extract was found deficient of artemisinin only. None of the standard compounds were detected in DW extract.

   2. Detection of polyphenolic compounds

      Quantification of polyphenolics as well as chromatographic fingerprinting of bioactive samples was done by RP-HPLC profiling using 8 standards (Fig. 2a). Quantities determined have been presented in Table 2. Significant quantities (\( \mu \)g/mgE) of vanillic acid (0.4 ± 0.01), syringic acid (0.1 ± 0.0), emodin (1.4 ± 0.13) and luteolin (0.7 ± 0.02) were retrieved in EA extract. A significant amount

### Table 1

| S. No. | Solvent Code | Polarity Index | Extract Yield (%) |
|-------|--------------|----------------|-------------------|
| 1     | NH           | 0              | 1.5\(^d\)          |
| 2     | EA           | 4.4            | 2.75\(^b\)         |
| 3     | M            | 5.1            | 4.25\(^b\)         |
| 4     | DW           | 9              | 5.95\(^b\)         |

Values are presented as mean from triplicate investigation. Means with different superscript (a–d) letters in the column are significantly (\( p < 0.05 \)) different from one another. NH: n-hexane, EA: Ethyl acetate, M: Methanol, DW: Distilled water.
of gallic acid has been quantified in M and DW extracts, i.e., 1.9 ± 0.03 and 0.1 ± 0.02 \( \mu g/mgE \), respectively (Fig. 2b).

### 3.2.4. High resolution mass spectroscopy

Polar extracts were selected for HRMS and their spectrum results are as shown in Fig. 3. Total 40 compounds were detected where M contained 25 compounds eluted between 0.38 and 8.56 min (Table 3). Of these 25 compounds, there were caffeoylquinic acid derivatives, cinnamic sucrose esters, esterified fatty acid, flavonoid glycosides, sesquiterpenoids, steroid, heneicosane derivative and sugar molecules. A single glycon part of a glycoside was also detected. DW extract had 15 compounds including flavonoid glycosides, caffeoylquinic acid derivatives, jasmonic acid, eudesmaneglucoside, neolignans glucoside, quinones, sesquiterpenoid derivative, triterpene derivative and steroid (cyasterone). Identification of these moieties was based on comparison of their relative retention times and mass spectra with those obtained from authentic sample literature survey.

### 3.3. Biological evaluation

#### 3.3.1. Antioxidant potential of \( A. \) \( \text{brevifolia} \) extracts

**3.3.1.1. Free radical scavenging assay.** Significant percent scavenging activity of \( A. \) \( \text{brevifolia} \) extracts was assessed by the discoloration of DPPH solution. The M and DW extracts depicted maximum \( (p < 0.05) \) scavenging potential (Table 4). EA did not exhibit any significant scavenging activity whereas minimal scavenging activity was shown by NH extract. IC50 of ascorbic acid was found to be 21.8 \( \mu g/ml \).

**3.3.1.2. Determination of total antioxidant capacity.** Total antioxidant capacity of the extracts were calculated by calibration curve \( y = 0.0268x - 0.062 \), \( R^2 = 0.9882 \) (Table 4). It was observed

### Table 2

RP-HPLC-DAD analysis of \( A. \) \( \text{brevifolia} \) extracts for their sesquiterpenes and polyphenolic composition.

| Standards (\( \mu g/mg \) of the extract) | Retention time (min) | Detection wavelength (nm) | Samples |
|------------------------------------------|-----------------------|---------------------------|---------|
| **Artemisinin and its analogues quantified in \( A. \) \( \text{brevifolia} \) extracts** | | | | |
| Artesunate | 6.7 | 210 | NH | 34.7 ± 0.01\(^b\) | 155 ± 0.20\(^a\) | 4.3 ± 0.07\(^c\) |
| DH Artemisinin | 9.3 | 210 | EA | 643 ± 0.19\(^b\) | 895 ± 0.03\(^a\) | 63.7 ± 0.04\(^c\) |
| Artemisinin | 12.9 | 210 | M | 15.2 ± 0.11\(^b\) | 50.7 ± 0.01\(^a\) |
| Artemether | 20.9 | 210 | DW | 49.2 ± 0.31\(^b\) | 81.4 ± 0.3\(^a\) | 9.68 ± 0.10\(^b\) |
| **Phenolic composition (\( \mu g/mg \) of the extract) of \( A. \) \( \text{brevifolia} \) extracts** | | | | |
| Vanillic acid | 9.517 | 257 | NH | *** | 0.4 ± 0.01\(^a\) |
| Gallic acid | 3.434 | 279 | EA | *** | 1.9 ± 0.03\(^a\) |
| Syringic acid | 9.515 | 279 | M | *** | 0.1 ± 0.02\(^a\) |
| Emodin | 27.844 | 279 | DW | *** | 1.4 ± 0.13\(^a\) |
| Luteolin | 18.372 | 325 | | *** | 0.7 ± 0.02\(^a\) |

Values are presented as mean ± SD from triplicate investigation. Means with different superscript \( (a–c) \) letters in the row are significantly \( (p < 0.05) \) different from one another. \( _{–} \): not detected, \( ^{**}_{–} \): not evaluated, NH: n-hexane, EA: Ethyl acetate, M: Methanol, DW: Distilled water.

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**Fig. 1.** RP-HPLC chromatograms of \( A. \) \( \text{brevifolia} \) extracts for artemisinin and its derivatives. i: standard compounds, ii: NH, iii: EA and iv: M extracts of \( A. \) \( \text{brevifolia} \) showing the presence of artemisinin and derivatives. 1: artesunate, 2: dihydro artemisinin, 3: artemisinin, 4: artemether. NH: n-Hexane, EA: Ethyl acetate, M: Methanol, DW: Distilled water.
that highest antioxidant capacity \( (p < 0.05) \) was depicted by M extract while NH exhibited minimum capacity. Total antioxidant capacity of \( A. \) brevifolia extracts was found to decrease in the following order M > DW > EA > NH.

3.3.1.3. Determination of total reducing power. Total reducing power of the extracts were calculated by calibration curve \( y = 0.0284x + 0.0247, R^2 = 0.9965 \) (Table 4). Assay results showed that M extract exhibited maximum \( (p < 0.05) \) reduction potential \( (112.3 \pm 1.2 \ \mu g) \).
AAE/mgE). DW extract also exhibited significant TRP followed by EA extract. Least TRP was manifested by NH extract, i.e., 16.6 ± 0.6 μg AAE/mgE.

3.3.1.4. β-carotene scavenging activity. It is quite evident from the results that M extract showed the minimum IC50 value, i.e., 45.44 ± 1.23 μg/ml, as compared to other samples (Table 4). DW also exhibited strong antioxidant potential. Non-polar extracts, i.e., NH and EA exhibited an IC50 greater than 200 μg/ml. Catechin was used as a positive control, which gave an IC50 value of 7.87 ± 0.15 μg/ml.

3.3.1.5. Iron chelating activity. Minimum IC50 values for iron chelation (28.21 ± 1.18 and 45.33 ± 2.06 μg/ml) were demonstrated by M and DW extracts (p < 0.05), respectively (Table 4). Herein, EA and NH extracts also exhibited a moderately significant activity, i.e., IC50 139.45 ± 0.97 and 165.74 ± 2.76 μg/ml. Iron chelation potential of M extract was comparable to that exhibited by EDTA (18.85 ± 1.53 μg/ml; positive control).

3.3.2. Antimicrobial potential of A. brevifolia extracts

(1) Antibacterial assay

Results clearly depict that EA extract was found remarkably active (p < 0.05) against all the tested bacterial strains (Table 5). Maximum activity was found against E. coli, B. subtilis, P. aeruginosa and MRSA (MIC = 22.22 μg/ml). NH extract also demonstrated toxicity against all bacterial strains except Resistant S. hemolyticus. M extract showed maximum antibacterial activity against E.coli (MIC 22.22 μg/ml), P. aeruginosa, Resistant P. aeruginosa and MRSA (MIC 66.66 μg/ml). DW was active against E. coli, and P. aeruginosa (MIC 66.66 and 200 μg/ml, respectively). DMSO did not show any activity.

(2) Antifungal assay

EA extract exhibited significant (p < 0.05) antifungal potential against F. solani and A. fumigatus with MIC values of 66.66 and 22.22 μg/ml, respectively (Table 5). M extract also demonstrated activity against A. flavus and A. fumigatus, MIC = 66.66 μg/ml. A moderately significant activity against F. solani, with an inhibition zone of 10 ± 0.67 mm was exhibited by DW extract. NH extract did not show any activity. DMSO was also found inactive.

3.3.3. Antiprotozoal potential of A. brevifolia extracts

(1) Antileishmanial activity

It is quite evident from the results that NH and EA extracts demonstrated a significant (p < 0.05) antileishmanial potential with IC50 values of 13.91 ± 0.04 and 15.40 ± 0.09 μg/ml, respectively (Fig. 4). M and DW extracts also exhibited an inhibition of 48.57 ± 1.21 and 26.56 ± 0.41%, respectively. Amphotericin B gave an IC50 value of 0.01 μg/ml while no activity was observed with DMSO.
(2) Antimarial activity

Herein, EA extract showed noteworthy \((p < 0.05)\) antimarial potential with an IC\(_{50}\) of 3.5 \(\pm\) 0.07 and 9.02 \(\pm\) 0.39 \(\mu\)g/ml, against D6 and W2, respectively \((\text{Fig. 4})\). Rest of the samples also manifested a moderately significant antimarial potential. Minimum antimarial potential was exhibited by DW extract. DMSO did not show any inhibition. (Table 3).

3.3.4. Cytotoxic potential of \(A.\) brevifolia extracts

(1) Cytotoxicity against \(A.\) salina \((\text{Brine shrimps})\)

All the tested manifested notable \((p < 0.05)\) shrimp's lethality potential \((\text{Table 6})\). EA extract was found to be highly potent with LD\(_{50}\) of 25 \(\pm\) 1.9 \(\mu\)g/ml. It was followed by NH and M extracts. Minimum cytotoxic potential was exhibited by DW extract with LD\(_{50}\) 40.3 \(\pm\) 3.7 \(\mu\)g/ml. Positive control used was doxorubicin, which demonstrated the LD\(_{50}\) 5.98 \(\pm\) 0.2 \(\mu\)g/ml whereas DMSO did not reveal any toxicity.

(2) Cytotoxicity against cancer cell lines

Almost all the extracts demonstrated substantial \((p < 0.05)\) cytotoxic potential against tested human cancer cell lines \((\text{Table 6})\). Among all the extracts, EA showed a comparatively significant \((p < 0.05)\) potential, against all the cell lines except MCF-7. NH and M extracts were active against HepG2 and DU-145 cell lines \((\text{IC}\(_{50}\) 19.6 \(\pm\) 11; 19.0 \(\pm\) 0.2 and 18.66 \(\pm\) 2.9; 18.82 \(\pm\) 0.4 \(\mu\)g/ml,
Table 4
Phytochemistry, antioxidant and in vitro anti-inflammatory potential of A. brevifolia extracts.

| Samples | Phytochemical assays | Antioxidant assays | In vitro anti-inflammatory assay |
|---------|----------------------|--------------------|---------------------------------|
|         | TPC | TFC | TAC | TRP | FRSA | β-carotene bleaching potential | Iron chelating potential | Nitric oxide scavenging potential |
|         | µg GAE/mgE | µg QE/mgE | µg AAE/mgE | µg AAE/mgE | POI | IC₅₀ (µg/ml) | IC₅₀ (µg/ml) | IC₅₀ (µg/ml) |
| NH      | 1.42 ± 0.03⁸ 1.62 ± 0.02⁸ | 14.2 ± 0.4⁸ | 16.6 ± 0.6⁸ | 11.5 ± 0.06⁸ | -200⁸ | 165.74 ± 2.76⁸ | >200⁸ |
| EA      | 4.2 ± 0.03⁹ 2.82 ± 0.29⁹ | 19.72 ± 0.8⁹ | 25.4 ± 1.0⁹ | 12.28 ± 2.6⁵ | -200⁹ | 139.45 ± 0.97⁹ | >200⁹ |
| M       | 16.96 ± 0.12⁵ 10.30 ± 0.48⁵ | 65.7 ± 0.4⁵ | 112.3 ± 1.2⁵ | 85.75 ± 0.3⁸ | 21.15 ± 0.01⁸ | 45.44 ± 1.2³ | >200⁶ |
| DW      | 10.25 ± 0.28⁶ 7.26 ± 0.02⁶ | 43.45 ± 0.42⁶ | 78.9 ± 2.3⁶ | 81.01 ± 0.18⁶ | 21.68 ± 0.04⁶ | 52.03 ± 2.56⁶ | >200⁰ |

Results presented are the mean ± SD of triplicate analysis. Means with different superscript (a–d) letters in the column are significantly (p < 0.05) different from one another.

—: No activity detected, ***: Not evaluated, NH: n-hexane, EA: Ethyl acetate, M: Methanol, DW: Distilled water, POI: Percent of inhibition, TPC: Total phenolic content, TFC: Total flavonoid content, TRP: Total reduction potential, FRSA: Free radical scavenging assay.

respectively). DW extract was found active against THP-1 cell line with an IC₅₀ value of 18.6 ± 1.9 µg/ml.All the extracts were found most active against THP-1 cell line. The cytotoxic potential of A. brevifolia was evaluated for the first time. DMSO did not exhibit cytotoxicity.

(3) Cytotoxicity against isolated lymphocytes

Cytotoxic potential of the extracts was also evaluated against isolated lymphocytes at similar concentration used for cytotoxicity assessment against cancer cell lines (Table 6). Data clearly indicates that none of the extracts exhibited toxicity against the isolated lymphocytes. Vincristine, showed significant cytotoxicity against the isolated cells with IC₅₀ value of 6.7 ± 1.5 µg/ml.

3.3.5. Genotoxicity assays

(1) Comet assay

Maximum DNA damage was produced by cisplatin (p < 0.05). Herein, %DNA migration of 47.7 ± 1.4%, 50.8 ± 1.4%, 50.7 ± 1.7% and 45.9 ± 0.7 % of comet’s tail was observed in case of HepG2, THP-1, HT-29 and DU-145 cell lines, respectively (Table 7). Extracts did not induce DNA damage, as %tail DNA ranges from 3.17 ± 0.1 to 7.69 ± 0.2% in different cancer cell lines, exhibiting the safe behavior of A. brevifolia. Negative control also did not depict genotoxicity as 5.36 ± 0.1% was the maximum %tail DNA observed in case of DMSO (Fig. 5).

(2) Micronucleus assay

Effects of four different extracts of A. brevifolia on %MN formation per 1000 BN cells and NDI values have been presented in Table 7. Cisplatin significantly (p < 0.05) increased the chromosomal damage in all the cell lines with minimum %MN formation observed in DU-145 cell lines (22.5 ± 2%). It is quite evident from the results that extracts did not induce any significant MN formation as compared to intact control. DMSO also did not induce any DNA damage. Maximum %MN formation, in case of DMSO (1.83 ± 0.2%) was observed in HepG2 cells. While measuring the second parameter which is NDI, CP alone insignificantly decreased NDI when compared to the untreated cells, i.e., 1.03 ± 0.1, 1.05 ± 0.2, 1.12 ± 0.1 and 1.01 ± 0.1 in HepG2, THP-1, HT-29 and DU-145 cell lines. The NDI was comparatively higher in extracts treated cells as compared to cells treated with positive control. Results clearly indicate that A. brevifolia extracts did not induce genotoxicity.

(3) Chromosomal aberration assay

Anticlastogenic effect of the extracts was observed through chromosomal aberration assay (Table 7). The number of aberrant metaphases (NAMP) observed in cisplatin treated cells were significantly (p < 0.05) higher than extracts treated cell lines. In case of HepG2 cells, maximum NAMP (38) was observed in cisplatin treated cells whereas within the extracts treatment it ranges from 1 to 3. DMSO did not induce any significant aberration as total 2 aberrations were observed. Similar was the case observed in other cell lines, i.e., THP-1, HT-29 and DU-145 where total NAMP, within the extracts treated groups, ranges from 1 to 3, 1 to 2 and 1 to 3, respectively. A noteworthy difference in the total number of chromosomal aberrations (TNCA), in comparison to cisplatin, was observed. TNCA was found to be maximum in CP treated cells, i.e., 57, 58, 59 and 52 in HepG2, THP-1, HT-29 and DU-145 cell lines, respectively. Maximum TNCA in NH treated cells was 2, which was observed in HepG2 cell lines whereas it was 3 (HepG2 cell lines) in case of EA extract, 2 (all the cell lines tested) in case of M extract and 2 (HepG2 and HT-29 cell lines) in case of DW extract. A significant reduction in the key cytogenetic parameters was also revealed.

3.3.6. Anti-inflammatory assays

(1) In vitro nitric oxide scavenging assay

A significant NO scavenging activity was depicted by all the extracts (Table 4). Maximum scavenging potential (p < 0.05) was shown by M extract (IC₅₀ 19.91 ± 0.23 µg/ml) followed by DW and EA extracts, respectively. NH exhibited the least scavenging with IC₅₀>200 µg/ml. Ascorbic acid (positive control) exhibited an IC₅₀ of 5.47 ± 0.38 µg/ml whereas DMSO did not show any activity.

(2) In vivo carrageenan induced paw edema inhibition assay

Mostly tested exhibited highest effects in 4th h of treatment (Fig. 6). Among all extracts, M extract showed significant (p < 0.05) inhibition of edema at high dose of 300 mg/kg BW, i.e., 30.1 ± 2.4, 50 ± 2.1, 65.7 ± 2.12 and 77.22 ± 1.9% in comparison to ibuprofen (standard drug used). Ibuprofen exhibited an edema inhibition of 18.8 ± 0.93, 49.59 ± 1.8, 77.67 ± 2.9 and 90.52 ± 2.95% at 1st, 2nd, 3rd and 4th h, respectively. Inhibition potential of edema decreased in the following trend among the extracts; MH > ML > DWH > DWL > EAH > EAL > NHH > NHL.
Table 5
Antibacterial and antifungal activities of *A. brevifolia* extracts.

| Samples | Antibacterial potential | Antifungal potential |
|---------|--------------------------|----------------------|
|         | Diameter of zone of inhibition (ZOI: mm) & MIC (µg/ml) |                     |
|         | Strains | ZOI | MIC | Strains | ZOI | MIC | Strains | ZOI | MIC | Strains | ZOI | MIC | Strains |
| NH K. pneumoniae | 17 ± 0.2<sup>c</sup> | 200<sup>a</sup> | 66.66<sup>b</sup> | Methicillin Resistant | 17 ± 0.56<sup>c</sup> | 66.66<sup>b</sup> | Mucor Spp. | — | *** | A. flavus | — | *** |
| EA | 18 ± 0.4<sup>a</sup> | 200<sup>a</sup> | 22.22<sup>b</sup> | S. aureus | 22 ± 0.34<sup>a</sup> | 22.22<sup>b</sup> | — | *** | — | *** |
| M | 13 ± 2.2<sup>b</sup> | 200<sup>a</sup> | 9 ± 0.87<sup>b</sup> | — | 19 ± 0.52<sup>b</sup> | 66.66<sup>c</sup> | — | *** | — | *** |
| DW | 11 ± 3.3<sup>c</sup> | — | 10 ± 0.5<sup>d</sup> | — | 9 ± 0.23<sup>d</sup> | — | — | *** | — | *** |
| Cefixime | 20 ± 2.2<sup>b</sup> | 1.11<sup>b</sup> | — | *** | — | *** | — | *** | — | *** |
| Roxithromycin | 22 ± 0.6<sup>e</sup> | — | 1.11<sup>c</sup> | — | *** | — | *** | — | *** |
| Clotrimazole | *** | *** | — | *** | — | *** | — | *** | — | *** |
| DMSO | — | — | — | — | — | — | — | — | — | — |
| NH S. aureus | 20 ± 0.3<sup>c</sup> | 22.22<sup>b</sup> | 18 ± 0.3<sup>a</sup> | F. solani | 17 ± 0.12<sup>c</sup> | 66.66<sup>b</sup> | A. fumigatus | — | *** | — | *** |
| EA | 19 ± 0.5<sup>a</sup> | 66.66<sup>a</sup> | 21 ± 0.3<sup>d</sup> | Resistant E. coli | 14 ± 0.15<sup>c</sup> | 200<sup>a</sup> | — | *** | — | *** |
| M | 7 ± 0.21<sup>d</sup> | — | 16 ± 0.2<sup>a</sup> | Resistant P. aeruginosa | 15 ± 0.63<sup>b</sup> | 200<sup>a</sup> | — | *** | — | *** |
| DW | 7 ± 0.5<sup>d</sup> | — | 12 ± 0.9<sup>d</sup> | 200<sup>a</sup> | 10 ± 0.42<sup>e</sup> | — | 10 ± 0.67<sup>c</sup> | *** | — | *** |
| Cefixime | *** | *** | 22 ± 0.8<sup>b</sup> | 11 ± 1.5<sup>e</sup> | *** | *** | *** | *** | *** | *** |
| Roxithromycin | 23 ± 0.5<sup>c</sup> | 1.11<sup>c</sup> | — | *** | — | *** | — | *** | — | *** |
| Clotrimazole | *** | *** | — | *** | — | *** | — | *** | — | *** |
| DMSO | — | — | — | — | — | — | — | — | — | — |
| NH E. coli | 21 ± 0.2<sup>d</sup> | 22.22<sup>b</sup> | 20 ± 0.5<sup>a</sup> | A. niger | 11 ± 0.44<sup>c</sup> | — | 7 ± 0.31<sup>c</sup> | *** | — | — |
| EA | 20 ± 0.3<sup>d</sup> | 22.22<sup>b</sup> | 19 ± 0.2<sup>d</sup> | S. hemolyticus | 19 ± 0.83<sup>c</sup> | 66.66<sup>b</sup> | — | *** | — | *** |
| M | 25 ± 0.3<sup>d</sup> | 22.22<sup>b</sup> | 19 ± 0.7<sup>d</sup> | 66.66<sup>a</sup> | 12 ± 0.32<sup>b</sup> | 200<sup>a</sup> | — | *** | — | *** |
| DW | 15 ± 0.7<sup>c</sup> | 66.66<sup>a</sup> | 10 ± 0.5<sup>d</sup> | — | 9 ± 0.29<sup>d</sup> | — | 8 ± 0.32<sup>b</sup> | *** | — | *** |
| Cefixime | 26 ± 3.5<sup>c</sup> | 1.11<sup>c</sup> | — | *** | — | *** | — | *** | — | *** |
| Roxithromycin | *** | *** | — | *** | — | *** | — | *** | — | *** |
| Clotrimazole | — | — | — | — | — | — | — | — | — | — |

Values presented are the mean ± SD of triplicate analysis. Means with different superscript (a–e) letters in the row are significantly (*p* < 0.05) different from one another. —: No activity detected, ***: Not evaluated, NH: n-hexane, EA: Ethyl acetate, M: Methanol, DW: Distilled water, DMSO: Dimethyl sulfoxide.
Table 6

Cytotoxic potential of extracts. *A. brevifolia*, isolated lymphocytes & cancer cell lines

| Sample          | LD50 (mg/ml) | IC50 (mg/ml) | %In |
|-----------------|--------------|--------------|-----|
| Lymphocytes     |              |              |     |
| A. salina       | NH           | 27.7 ± 0.9   | 5.4 ± 1.6 | >20a |
|                 | EA           | 25 ± 1.9     | 6.3 ± 1.3 | >20a |
|                 | DW           | 40.3 ± 3.7   | 12.1 ± 0.6 | >20a |
|                 | DR           | 5.98 ± 0.2   | 77.4 ± 3.7 | >20a |
|                 | VC           | 6.7 ± 1.3   | 205.7 ± 0.5 | >20a |
|                 | CP           | 97.4 ± 0.2   | 205.7 ± 0.5 | >20a |
|                 | DMG2         | -            | -         | -    |
| HepG2           |              |              |     |
| A. salina       | NH           | 18.8 ± 2.1   | 4.1 ± 1.4 | >20a |
|                 | EA           | 14.5 ± 0.2   | 5.1 ± 0.9 | >20a |
|                 | DW           | 36.4 ± 0.7   | 18.1 ± 0.9 | >20a |
|                 | DR           | 98.9 ± 0.2   | 17.1 ± 0.2 | >20a |
|                 | VC           | 99.2 ± 0.3   | 0.76 ± 0.1 | >20a |
|                 | CP           | 99.4 ± 0.5   | 0.05 ± 0.5 | >20a |
|                 | DMG2         | -            | -         | -    |
| THP-1           |              |              |     |
| A. salina       | NH           | 18.2 ± 0.8   | 4.4 ± 0.9 | >20a |
|                 | EA           | 14.1 ± 0.9   | 5.2 ± 0.7 | >20a |
|                 | DW           | 35.3 ± 5.2   | 18.5 ± 0.9 | >20a |
|                 | DR           | 99.3 ± 0.9   | 0.9 ± 0.1 | >20a |
|                 | VC           | 99.2 ± 0.3   | 0.3 ± 0.1 | >20a |
|                 | CP           | 99.4 ± 0.5   | 0.05 ± 0.5 | >20a |
|                 | DMG2         | -            | -         | -    |
| MCF-7           |              |              |     |
| A. salina       | NH           | 18.2 ± 0.8   | 4.4 ± 0.9 | >20a |
|                 | EA           | 14.1 ± 0.9   | 5.2 ± 0.7 | >20a |
|                 | DW           | 35.3 ± 5.2   | 18.5 ± 0.9 | >20a |
|                 | DR           | 99.3 ± 0.9   | 0.9 ± 0.1 | >20a |
|                 | VC           | 99.2 ± 0.3   | 0.3 ± 0.1 | >20a |
|                 | CP           | 99.4 ± 0.5   | 0.05 ± 0.5 | >20a |
|                 | DMG2         | -            | -         | -    |

Results are presented as mean ± SD (n=3). Student’s t-test with different superscripts (a-d) between two groups are significantly (p < 0.05) different from one another. — No activity detected. **Not evaluated. NH: n-hexane, EA: Ethyl acetate, M: Methanol, DW: Distilled water, CP: Cisplatin, DR: Doxorubicin, VC: Vincristine, 5-FU: 5-Fluorouracil, DMSO: Dimethly sulfoxide.

(3) *In vivo croton oil induced anal edema inhibition assay*

Results showed that polar extracts demonstrated a significant (p < 0.05) activity in comparison to nonpolar extracts (Fig. 6). Highest anti-inflammatory potential was revealed by DW extract (74. 44 ± 0.96%) as compared to ibuprofen (86.85 ± 2.4%). It was followed by M (57.22 ± 2.5%), EA (52.04 ± 0.71%) and NH (33.33 ± 1. 01%) extracts. Vehicle control did not induce any activity.

3.3.7. Enzyme inhibition assays

(1) α-amylase inhibition assay

Significant (p < 0.05) carbohydrate-metabolizing enzyme inhibition was exhibited by DW and M extractswith IC50 values of 89.7 ± 1.08 and 105.74 ± 1.43 µg/ml, respectively (Fig. 7). Rest, EA and NH extracts depicted amoderate enzyme inhibition, i.e., 42.21 ± 0.987% and 33.91 ± 0.987%, respectively. Acarbose, the positive control used, exhibited an IC50 value of 33.73 ± 0.12 µg/ml.

(2) α-glucosidase inhibition assay

Inhibition of carbohydrate-metabolizing enzymes was further investigated by α-glucosidase inhibition assay (Fig. 7). The DW extract showed notable (p < 0.05) α-glucosidase inhibition (IC50 value of 98.14 ± 0.47 µg/ml). Minimal enzyme inhibition was exhibited by M, NH and EA extracts, respectively.

4. Discussion

*Artemisia brevifolia* is one of the pharmacologically active plants of genus *Artemisia* that has been used in traditional medicine to treat various ailments. Despite its effectiveness, scientific evidence proving its medicinal properties, is insufficient. Thus, in the current study, we evaluated pharmacological potential of *Artemisia brevifolia* via a battery of assays. Initially, different extracts were prepared by successive extraction using solvents of variable polarity. Extraction is considered a potential limiting step while preparing plants’ samples for screening of the desired bioactivities (Ahmed et al., 2017). Its efficiency is usually affected by the nature of the solvent, solvent-to-solid ratio, particle size of grounded plant material, extraction duration and temperature. Extraction efficiency can be augmented by ultrasonic-assisted maceration (UAM) that accelerates solvent diffusion and dissolution of the solute. Furthermore, a significant (p < 0.05) increment in the extraction yield has been observed when solvents of different polarity are used, from non-polar to polar edge (Zahra et al., 2017). Both UAM and variable solvent polarity aided in better extract recovery in our study providing a range of phytoconstituents.

Next, phytoconstituents particularly polyphenolic compounds, artemisinin and its derivatives were quantified in extracts. Results clearly indicate that polar solvents, i.e., M and DW, exhibited better yield of total phenolic and flavonoid compounds, thus signifying the role of extraction solvents’ polarity (Table 4). These findings are in agreement with previous studies where significant amount of phenols were quantified in methanol extracts of *A. campestris* (Mourad et al., 2018). Iqbal et al. (2012) also reported polar solvents as better extraction media for flavonoids. The conjugation of (Mourad et al., 2018). Iqbal et al. (2012) also reported polar solvents as better extraction media for flavonoids. The conjugation of methoxy groups and benzo-y-pyrone ring, impart credible antioxidant properties either by free radical scavenging, singlet oxygen...
traditional Chinese medicine for their respective immunostimulant(s) evinced significant amounts of emodin, luteolin, vanillic acid, pestris, A. spicigera and A. splendens belonging to genus antitumor, and antidepressant activities (Fatima et al., 2015, Nasir ties, e.g., anti-inflammatory, antiulcer, antioxidant, antispasmodic, lipid peroxidation. These exhibit diverse pharmacological proper-
quenching, metal ion chelation, hydrogen donation or inhibition of lipid peroxidation. These exhibit diverse pharmacological properties, e.g., anti-inflammatory, antiuciel, antioxidant, antispasmodic, antitumor, and antidepressant activities (Fatima et al., 2015, Nasir et al., 2017). Flavonoids are the major phytonutrients of species belonging to genus Artemisia, i.e., A. vulgaris, A. absinthium, A. campestris, A. spicigera and A. splendens (Iqbal et al., 2012).

Chromatographic finger printing through RP-HPLC-DAD analysis evinced significant amounts of emodin, luteolin, vanillic acid, syringic acid in EA extract. These compounds have been used in traditional Chinese medicine for their respective immunostimulant (Zahra et al., 2017), enzyme inhibitory, genoprotective, antithrombotic (Fatima et al., 2015) and antiendotoxic (Srinivasulu et al., 2018) properties. Gallic acid, detected in M and DW extracts, has well reputed antioxidant, immuno-modulatory and antileishmanial potential (Fatima et al., 2015). Furthermore, artemisinin, dihydroartemisinin, artesunate and arteether were found in NH, EA and M extracts of A. brevifolia. Maximum amount of these antimalarial compounds was quantified in EA extract. The ß-lactone ring and endoperoxide bridge in the structure of these compounds make them weakly polar (Liu et al., 2009). On the basis of “like dissolve like” ethyl acetate (weakly polar solvents) extracted more amounts are compared to other solvents. Thus, here we present EA extract of A. brevifolia. An ample source of artemisinin and its derivatives.

HRMS analysis of A. brevifolia extracts evinced 40 chemical compounds in M and DW extracts. Polyphenolic compounds, sesquiterpenoids and lignin glycosides served as major phytonutrients of M extract whereas former class was dominant in DW extract. Results endorse better solubility of both of these chemical classes in polar solvents. These compounds have reported antiosteoporotic, anti diabetic, hepatoprotective (Nasir et al., 2020), antimalarial, antileishmanial (Rohmer 1999) and anticancer potential. Lignin glycosides were found active against different human breast cancer cell lines (BT549, MCF7 and MDA-MB-231) (Baek et al., 2018). A straight chain alkane, heptacosane derivative, was also detected in DW extract. This compound is reported to attract mosquitoes in lower concentration whereas at higher concentration they are repelled instead (Seenivasagan et al., 2009). Significant (p < 0.05) pharmacological potential of A. brevifolia extracts observed in the current study might be attributed to the presence of these compounds.

The pharmacological profile of A. brevifolia was established by antioxidant, anti-inflammatory, antileishmanial, antimalarial, antibacterial, antifungal and cytotoxic activities. Yin-yang, a complex relational concept in traditional Chinese medicine, expounds the importance of balance between oxidants and antioxidants. Oxidants, generated as byproducts at physiological concentrations, are required for numerous cellular functions. However, disproportionation production induces oxidative stress, which impair the integrity of vital biomolecules and triggers numerous ailments (Kazmi et al., 2018). Supplementing with exogenous natural antioxidants is plausible (Majid et al., 2018). Currently, M extract of A. brevifolia depicted maximum FRSA. M and DW extracts demonstrated highest antioxidant potential while evaluated through a battery of antioxidant assays. Results are in accordance with previous studies where methanol extract of A. absinthium (EREL et al., 2012), A. annua leaves (Iqbal et al., 2012) and polar extracts of A. campestris (Akrout et al., 2011) showed significant total antioxidant capacity, free radical scavenging and ß-carotene scavenging potential. Currently, a number of polyphenolic compounds have been detected in A. brevifolia extracts. Significant (p < 0.05) antioxidant assertiveness might be accredited to the presence of these compounds. These compounds exert the antioxidant effects by virtue of the hydroxyl groups (Kazmi et al., 2018). In addition, total reduction potential of A. brevifolia extracts might be attributed to the presence of artemisinin, which is known to reduce ferric ions to ferrous (Sibmooh et al., 2001).

Iron chelating assay involves the sample’s ability to chelate with iron (II), thus hampering the iron-ferrozine complex formation and lowering color intensity of the solutions. Our findings are in consensus with that of Majid et al. (2015) as M and DW extracts exhibited significant (p < 0.05) iron chelation potential in comparison to Catechins. In contrast to other antioxidant assays, herein, NH and EA extracts also gave moderately significant (p < 0.05) results. This potential might be associated to the presence of artemisinin and its analogues, as they chelate with iron to exert their antimalarial effect (O’Neill et al., 2010).

Subsequently, anti-infective activity of A. brevifolia extracts was determined against bacterial, fungal, malarial and leishmanial strains. Emergence of multidrug-resistant phenotypes of several pathogenic bacterial strains is a major health problem in the treatment of infections. Likewise, complications including hypersensitivity reactions and myelosuppression make it difficult to use certain antibiotics (Zahra et al., 2017). Current study presents A. brevifolia EA and NH extracts as possible source of antibacterial agents since they demonstrated significant (p < 0.05) inhibition of tested bacterial strains including P. aeruginosa and MRSA. Polyphenolic compounds inhibit microbial enzymes either by reacting with sulphydryl groups or by interacting with the proteins leading to microbial growth inhibition (Fatima et al., 2015). Moreover, sesquiterpenes are known to have strong antibacterial profile (Rohmer, 1999). Therefore, espied antimicrobial potential of the extracts can be ascribed to the presence of detected compounds (Rohmer, 1999).
Table 7
Effect of *A. brevifolia* extracts on DNA integrity of cancer cell lines.

| Cell lines employed | Comet assay | Micronucleus assay | Chromosomal assay | Exchange \(\text{dic} / \text{qr} / \text{Tr} / \text{DM} / \text{TNCA}\) |
|---------------------|-------------|--------------------|-------------------|---------------------|
| Samples             | CL (µm)     | HL (µm)            | TL (µm)           | %DIH                |
| **HepG2**           |             |                    |                   |                     |
| NH                  | 55 ± 0.5d   | 52 ± 0.4d          | 3 ± 0.8h          | 94.55 ± 0.8d        |
| EA                  | 52 ± 1.8e   | 50 ± 1.5j          | 2 ± 1.3d          | 96.15 ± 0.2b        |
| M                   | 62 ± 0.5b   | 59 ± 1.6h          | 3 ± 1.8g          | 95.16 ± 0.4h        |
| DW                  | 52 ± 1.8e   | 47 ± 1.3f          | 5 ± 0.9h          | 92.31 ± 0.5g        |
| IC                  | 61 ± 0.9h   | 58 ± 0.5j          | 3 ± 1.0l          | 95.08 ± 0.2d        |
| DMSO                | 62 ± 1.5b   | 61 ± 1.4a          | 1 ± 0.1k          | 98.39 ± 0.9a        |
| CP                  | 67 ± 1.3a   | 35 ± 2.5b          | 32 ± 1.4          | 52.23 ± 1.4a        |
| **THP-1**           |             |                    |                   |                     |
| NH                  | 52 ± 1.5k   | 50 ± 1.9b          | 2 ± 0.4q          | 96.15 ± 0.2b        |
| EA                  | 64 ± 1.3h   | 61 ± 1.9b          | 3 ± 0.4q          | 95.31 ± 1.9c        |
| M                   | 53 ± 1.2d   | 49 ± 2.0d          | 4 ± 0.9b          | 92.45 ± 2.4a        |
| DW                  | 58 ± 0.8j   | 55 ± 1.1d          | 3 ± 0.4g          | 94.83 ± 0.3l        |
| IC                  | 62 ± 3.4d   | 59 ± 2.4f          | 3 ± 0.3h          | 95.16 ± 1.4f        |
| DMSO                | 67 ± 0.4a   | 65 ± 1.4a          | 2 ± 1.9g          | 97.01 ± 0.2e        |
| CP                  | 63 ± 1.4a   | 31 ± 1.7g          | 32 ± 2.0          | 49.20 ± 2.2h        |
| **HT-29**           |             |                    |                   |                     |
| NH                  | 51 ± 0.3f   | 48 ± 3.1h          | 3 ± 0.8g          | 94.12 ± 1.9g        |
| EA                  | 56 ± 2.5d   | 53 ± 1.4f          | 3 ± 0.9g          | 94.64 ± 2.6h        |
| M                   | 63 ± 0.2e   | 61 ± 2.3b          | 2 ± 0.3g          | 96.83 ± 1.0a        |
| DW                  | 66 ± 3.2b   | 63 ± 2.9g          | 3 ± 0.2b          | 95.45 ± 1.8b        |
| IC                  | 56 ± 2.1d   | 53 ± 1.5a          | 3 ± 0.1h          | 94.64 ± 0.2e        |
| DMSO                | 54 ± 1.3j   | 52 ± 1.6d          | 2 ± 0.8h          | 96.30 ± 3.00        |
| CP                  | 69 ± 1.0h   | 34 ± 0.4f          | 35 ± 1.4          | 49.27 ± 1.5d        |
| **DU-145**          |             |                    |                   |                     |
| NH                  | 61 ± 2.3b   | 57 ± 0.9b          | 4 ± 1.2e          | 93.44 ± 0.7e        |
| EA                  | 56 ± 0.5d   | 52 ± 2.8e          | 4 ± 0.2g          | 92.86 ± 2.10        |
| M                   | 66 ± 1.7a   | 63 ± 2.2a          | 3 ± 0.9g          | 94.55 ± 0.4a        |
| DW                  | 59 ± 2.1i   | 56 ± 1.3c          | 3 ± 0.3h          | 94.92 ± 0.8b        |
| IC                  | 61 ± 1.1l   | 52 ± 3.3d          | 3 ± 0.2g          | 92.86 ± 2.6d        |
| CP                  | 61 ± 0.9b   | 33 ± 3.2f          | 28 ± 3.3          | 54.10 ± 2.8h        |

Results are presented as mean ± SD. Means with different superscript (a–g) letters, are significantly \(p < 0.05\) different from one another. CL: Comet length, HL: Head length, TL: Tail length, %DIH: Percent of DNA in head, %DIT: Percent of DNA in tail, TM: Tail moment, %MN: Percent of micronuclei/Binucleated cells counted, NDI: Nuclear division index, NAMP: No. of aberrant metaphases, g: Gap, b/f: Break/fragment, dic: Dicentric, qr: Quadriradial, Tr: Triradial, dmin: Double minute, TNCA: Total number of chromosomal aberrations, NH: n-hexane, EA: Ethyl acetate, M: Methanol, DW: Distilled water, IC: intact control, CP: Cisplatin, DMSO: Dimethyl sulfoxide.
found active against *A. fumigatus* activity against fungal strains. M extract demonstrated significant fungal potential (Rohmer 1999, Nasir et al., 2017). EA and M cosides, glucosinolates and flavonoids are known to exhibit anti-secondary metabolites including terpenes, phenolics, phenolic glycosides, glucosinolates and flavonoids are known to exhibit antifungal potential (Rohmer 1999, Nasir et al., 2017). EA and M extracts were found rich in sesquiterpenes and polyphenolic compounds, that might be responsible for their antifungal potential.

Another aspect of the study was to find an alternative source of antimalarial artemisinin. Malaria is among the top public health challenges that caused 5 million cases in WHO Eastern Mediterranean Region in 2019 (Organization 2016). It is a common concern in Pakistan during every rainy season where emergence of drug resistant *P. falciparum* poses great threat. Since, *A. annua*, a major source of artemisinin, shares the same family with *A. brevifolia*; therefore, extracts of the selected plant were tested against both chloroquine sensitive (D6) and resistant strains (W2) of *P. falciparum* through flow cytometry. Flow cytometry is superior to other conventional method in analyzing malarial infection associated blood parasitemia. Basically, red blood cells (RBCs) primarily lack DNA. In case of infection, parasite metabolizes hydroethidine into ethidium, a nucleic acid-binding fluorochrome. Therefore, counting the number of positively stained RBCs and comparing the ratio to the total number of RBCs represent blood parasitemia (Kaou et al., 2008). All the extracts of *A. brevifolia* have manifested a significant ($p < 0.05$) antimalarial potential with EA extract exhibiting the maximum. HPLC analysis of NH, EA and M extracts has confirmed the presence of artemisinin and its derivatives. These drugs usually act by endoperoxide moiety, which cleaves and generate free radicals ultimately killing the parasite by alkylation and inhibiting protein or nucleic acid synthesis (O’Neill et al., 2010). Significant ($p < 0.05$) antimalarial potential of *A. brevifolia* extracts might be accredited to the presence of artemisinin and its derivatives. A number of polyphenolic compounds have also been detected through HPLC and HRMS studies. Antioxidant potential of these compounds can inhibit hemoglobin formation letting heme free, which is very toxic for the malarial parasite (Abu-Lafi et al., 2020). Their presence also hinders the development of parasite resistance.

Next, effectiveness of extracts against leishmaniasis was tested. Leishmaniasis, a neglected vector transmitted disease, has 1–1.5 million incidences of cutaneous while 0.5 million incidences of visceral leishmaniasis annually. Variable effectiveness, unacceptable side effects and emerging resistance against available drugs make them substandard (Waseem et al., 2017). Plant-derived products have gained eminent ground in search for better, effective and preferably non-toxic leishmanicidal compounds. A number of plant secondary metabolites including luteolin, vanillic acid, quassin, berberine and artemisinin have established antileishmanial profile (Ahmed et al., 2017). The presence of these compounds in *A. brevifolia* justifies its evaluation for antileishmanial potential.

Maximum antileishmanial potential was demonstrated by NH and EA extracts, which were found rich in sesquiterpenes and polyphenolics. Cytotoxicity of artemisinin in leishmanial promastigotes is mediated by a series of events, which are initiated by the cleavage of its endoperoxide bridge. This leads to prodigal ROS generation with concomitant non-protein thiols diminution and alteration of the mitochondrial membrane potential. Leishmanial parasite is more prone to oxidative stress as it cannot express catalase or peroxidase, the major H$_2$O$_2$ metabolizing enzymes. Moreover, a sole mitochondrion serves as the putative ‘power-house’ of the parasite. Therefore, modulation of the mitochondrial trans-membrane potential or inhibition of its respiratory complex, render it more vulnerable to plant-derived antileishmanial agents. All of these events culminated in a caspase-independent death, which mimics apoptosis (De Sarkar et al., 2019). Studies have shown that Totum, an herbal formulation comprising gelatin capsules filled with *A. annua* leaf powder has shown moderate activity against *L. panamensis* amastigotes (Mesa et al., 2017). Furthermore, Biliaet al. (2008) has reported that only the organic extracts of *A. annua* arrested the growth of *L. donovani*. These support our results.
making us to infer that antileishmanial potential is more prominent in the non-polar extracts of *Artemisia* species.

Continuing the pharmacological profiling of *A. brevifolia*, in vitro cytotoxic activity was determined using brine shrimps, isolated lymphocytes and cancer cell lines. Cancer is a global threat that despite extensive research has no complete cure so far (Waseem et al., 2017). About 18 million new cases of cancer were reported in 2018 that are expected to climb to approximately 29–37 million over the next twenty years. Natural products by virtue of their biochemical specificity and chemical diversity are considered a valuable source for the discovery of novel anticancer leads. Also, according to WHO cancer report 2018, a patient burdened with drug-induced toxicity can get help from the complementary and alternative medicine (Sung et al., 2021).

![Graph](image)

**Fig. 6.** Percentedema inhibition by *A. brevifolia* extracts. Datavalues(mean ± SD) are average of triplicate analysis. a: Percent of paw edema inhibition, b: Percent of analedema inhibition, Vehicle: 1% Dimethyl sulfoxide, Ibup: Ibuprofen, NHL: Low dose (150 mg/kg) of N-hexane, NHH: High dose (300 mg/kg) of N-hexane, EAL: Low dose (150 mg/kg) of Ethyl acetate, EAH: High dose (300 mg/kg) of Ethyl acetate, ML: Low dose (150 mg/kg) of Methanol, MH: High dose (300 mg/kg) of Methanol, DWL: Low dose (150 mg/kg) of Distilled water, DWH: High dose (300 mg/kg) of Distilled water extracts of *A. brevifolia*. 
Initially, cytotoxic potential of *A. brevifolia* extracts was assessed against brine shrimps. A positive correlation between the brine shrimp cytotoxicity and human nasopharyngeal carcinoma cell lines (KB cell lines) has been reported thus making the brine shrimp cytotoxicity and human nasopharyngeal carcinoma cell lines (KB cell lines) isomers prospective targets for antitumor and anticancer activities (Hendra et al., 2021). In the recent study, all the extracts have exhibited a concentration dependent lethality with LD₅₀ less than 1000 µg/ml. According to the norms of assay, nearly all the extracts possess cytotoxic potential as an LD₅₀ less than 50 µg/ml. According to the norms of assay, nearly all the extracts possess cytotoxic potential as an LD₅₀ less than 1000 µg/ml (Fatima et al., 2015). So, the cytotoxic potential of *A. brevifolia* extracts was further gauged against human hepatocarcinoma, leukemia, colon, prostate, breast and ovarian cancer cell lines.

According to WHO cancer report 2018, most frequently diagnosed cancer is female breast cancer (11.6%) followed by colorectal cancers (10.2%). Prostate cancer, hepatoma and leukemia are amongst the most widespread cancers in the world. Cumulatively, these cancers correspond to 54% of all cancer cases and 58% of all deaths (Sung et al., 2021).

Herein, EA was most active against all the cancer cell lines followed by NH and M extracts. According to the RP-HPLC analysis in our study, artemisinin and its derivatives though present in NH and M extract, are more concentrated in EA extract. Artemisinin and its analogs have been reported to exert their antitumor effects by inhibiting cancer proliferation, metastasis, and angiogenesis (Crespo-Ortiz and Wei, 2012). Polyphenolics detected in the extracts, i.e., gallic acid, quinic acid, apigenin, emodin also have well known anticancer profile (Nasir et al., 2020). Therefore, it can be surmised that artemisinin and its derivatives as well as detected antioxidant moieties might be responsible for the significant (*p* < 0.05) anticancer activity of the extracts. Though, plants perceptibly possess therapeutically active moieties; however, safety of the active concentration of these therapeutic moieties must be the prime determinant. This pertinent data can be generated by evaluating toxicity against isolated lymphocytes, which is a suitable alternative of *in vivo* models due to their economic and ethical concerns (Ahmed et al., 2017). Consequently, cytotoxicity of the extracts was assessed against isolated lymphocytes to establish their safety profile. Results clearly indicate the non-toxic behaviour of extracts against the isolated lymphocytes indicating a discerning response that was observed only against cancer cell lines and preventing normal cells to get damaged. On the contrary, vincristine, the positive control used, exhibited significant (*p* < 0.05) cytotoxicity against lymphocytes.

Next, genotoxic evaluation of *A. brevifolia* was conducted using a standard test battery to assess cytogenetic damage. It has been reported that pyrrolizidine alkaloids produced by the plants belonging to Orhidaceae, Boraginaceae, Poaceae and Asteraceae families can damage DNA and other cellular components, which regulate the fidelity of the genome (Schramm et al., 2019). Such genotoxic agents interact with a specific DNA base sequence causing breakage, lesions, deletion, fusion, non-disjunction or segregation leading to damage and mutation. Therefore, testing at earlier stages is imperative for the hazard assessment of natural products to identify genotoxic liabilities. Different endpoints can be anticipated while evaluating genotoxicity, i.e., point mutations induction, changes in chromosome structure (breaks, deletions, rearrangements) or chromosomal number (polyplody or aneuploidy). No single test can envisage an unequivocal ruling about the genotoxic potential of any substance (Savale, 2018). Thus, comet, micronucleus and chromosomal aberration assays were performed on extracts of *A. brevifolia*.

Comet assay, being highly sensitive, detects single and double strand breaks, repair induced breaks, alkali labile lesions and abasic sites even at a single cell level and is called single-cell gel electrophoresis (Majid et al., 2018). Breakage of supercoiled DNA leaves it un-winded, which then extends out of the gel layer and appears as a comet under electrophoresis at high pH. Relative intensity of comets' tail DNA is directly proportional to DNA breakage frequency (Kazmi et al., 2018).

Currently, maximum DNA (*p* < 0.05) damage was produced by 5-Flourouracil, which is evident from the migration of 32.15% of DNA to comet's tail. On the contrary, the extracts showed minimal formation of comets’ tail. Furthermore, cytogenic damage was evaluated using micronucleus and chromosomal aberration assay. Micronucleus assay involves the identification of a small, membrane bounded DNA fragment in the cytoplasm of the cell called micronuclei, which may originate from acentric chromosome fragments or whole chromosomes. The increased micronuclei formation indicates the frequency and severity of DNA damage. Assay comprehensively investigate both aneugens and clastogens as well as other cellular and nuclear dysfunctions (Tsuhoy et al., 2007). It also measures the NDI, which itself is a measure of genomic aberration. The rationale behind NDI measurement is that the cells with greater chromosomal damage are less likely to enter cell division or cell death occurs before cell division. If the cells fail to divide during the cytokinesis-block phase, they will remain mono-nucleated with the lowest NDI value of 1.0. If binucleated cells are formed after completing one division, NDI will be 2.0 and so on (Ipek et al., 2017).

In current study, no significant percent of micronucleus formation was observed in extract groups and the NDI was signif-
icantly ($p < 0.05$) higher than positive control cisplatin. Subsequent
analysis of chromosomal aberration showed that the extracts
did not induce DNA damage. The cell lines were treated
with a direct mutagen, cisplatin, which is known to cause inter
and intrasand DNA crosslinks. It perturbs DNA replication
and transcription eventually leading to cell cycle arrest and cell
apoptosis. Drug develops structural aberrations by inducing
chromatid or chromosomal breaks (Imreova et al., 2017). Here,
an anticlasterogenic activity was revealed against cisplatin in
extract treated groups. It can be explained by the increased
detoxification or excessive DNA repair stimulation by virtue of
strong antioxidant assertiveness of the extracts (Kazmi et al.,
2018). These results showed that the extracts of A. brevilifolia
are not genotoxic, supporting their use by the natives of North-
ern areas of Pakistan. It can also be inferred that the extracts
have exerted their significant ($p < 0.05$) cytotoxic potential
through a mechanism other than DNA damage, which needs to
be investigated through further studies.

Natural products with antioxidant and cytotoxic potentials are
expected to have anti-inflammatory profile as well. This is based
on the fact that reactive oxygen species are the prime mediators
for oxidative stress and inflammation. Nitric oxide, an important
chemical mediator of oxidation and inflammation, is involved in
the regulation of numerous physiological processes but its exces-
sive production brings about deleterious effects (Faroq et al.,
2020). It reacts with oxygen under aerobic conditions and gener-
ates highly reactive intermediate species, i.e., nitrate, nitrite and
peroxynitrite, which can induce oxidative stress. Therefore, inhibi-
tion of NO production serves as a key factor for the screening of
drugs with anti-inflammatory potential (Fatima et al., 2020).
Moreover, many debilitating diseases including rheumatoid arthrits,
diabetes, cancer and depression root back to inflammatory pro-
cesses. Despite the availability of various anti-inflammatory drugs
in the market, there is an overall shift from synthetic drugs to nat-
ural therapy (Karimi et al., 2015). In line with this, we evaluated the
in vitro and in vivo anti-inflammatory activity of A. brevilifolia
extracts. M and DW extracts showed highest inhibition of NO. Fur-
thermore, results of in vivo paw andan dal edema models also pre-
presented M and DW extracts as potent anti-inflammatory agents as
compared to other extracts. Maximum phenolic and flavonoid con-
tents were preliminarily quantified in these extracts, which can be
related to their anti-inflammatory potential. Being rich in bioactive
polyphenolics, these extracts quenched NO thus halting the pro-
duction of highly reactive intermediates by consuming available
oxygen. Similarly, M and DW extracts were found to be rich in caf-
feoylquinic acids, apigenin, gallic acid and jasmonic acid. All of
these compounds have established anti-inflammatory profile
(Gutiérrez-Grijalva et al., 2018).

Lastly, the pharmacological evaluation of A. brevilifolia extracts
was extended to determine the inhibition of carbohydrate-
metabolizing enzymes by the extracts. Inhibitors of carbohydrate-
metabolizing enzymes $\alpha$-amylase and $\alpha$-glucosidase have offered new avenues for the management of
postprandial glucose blood level. Found in saliva and pancreatic
juice, $\alpha$-amylases hydrolyzes the large insoluble starch molecules
to soluble oligosaccharides, which are converted to glucose
molecules by the $\alpha$-glucosidases present in the brush-border sur-
face membrane of small intestine (Ahmed et al., 2019). This
leads to a rise in blood glucose levels, which can be debilitating
for patients of diabetes mellitus. Inhibition of $\alpha$-amylase and $\alpha$-
glucosidase enzymes can reduce the cleavage of complex carbo-
hydrates into glucose that will aid in the management of post-
prandial hyperglycemia in diabetes patients. Chronic hyperglycemia
augments the generation of ROS, accentuating the oxidative damage, which ultimately curtails the insulin
secretion with concurrent negative regulation of insulin signaling
cascade (Ahmed et al., 2017).

In the present work, DW and M extracts demonstrated a signif-
icant ($p < 0.05$) inhibition of $\alpha$-amylase and $\alpha$-glucosidase
enzymes. The $\alpha$-amylase inhibition potential of DW extract is
somewhat analogous to that of standard acarbose. Numerous
plant-derived secondary metabolites including phenolic com-
pounds, and flavonoids have been proven to possess $\alpha$-amylase
and $\alpha$-glucosidase inhibitory activities. In the current study, a sub-
stantial amount of phenolics and flavonoids including apigenin,
jasmonic acid and caffeoylquinic acid has been detected in DW
extract. Apigenin has been reported for its $\alpha$- amylase activity
(Wang et al., 2010). Previously, mono, di and tri-substituted caf-
feoylquinic acid were found to be the predominant phenolic group
of Siberian Artemisia species, responsible for $\alpha$-amylase and $\alpha$-
glucosidase inhibitory activities (Olennikov et al., 2018). Moreover,
studies have reported that jasmonic acid elicitation has improved
the anti-$\alpha$-amylase potential of several essential oils (Zlotek et
al., 2020). Mass spectroscopy of DW extract has revealed the
presence of these compounds. Therefore, substantial
carbohydrate-metabolizing inhibition by distilled water extract
might be accredited to the presence of these compounds.

5. Conclusions

In conclusion, the current study presents A. brevilifolia as a rich
source of artemisinin and its derivatives, which might have played
the key role in several of its pharmacological attributes, i.e., anti-
malarial, anticancer and antidiabetic activities. The EA extract
showed good antimalarial and antileishmanial activities especially
against resistant malarial strain. Manifestation of important sec-
ondary metabolites including phenolics and flavonoids supports
substantial antioxidant properties of the extracts. Significant
anti-inflammatory potential deemed these extracts as suitable can-
didates for detailed evaluation at molecular level. Noteworthy
anti-bacterial potential especially against resistant strains justifies
its ethnopharmacological use to treat a number of infections.
Moreover, the results of genotoxic assays confirm the safe behavior
of the plant and also helped to infer that the extracts have exerted
their significant cytotoxic potential through a mechanism other
than DNA damage, which needs to be investigated through further
studies.

Declaration of Competing Interest

The authors declare that they have no known competing finan-
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Availability of data

We hereby confirm that all the data produced in the manuscript is original based on extensive repeated experimentation and can be provided upon reasonable request, when needed.

Ethics approval and consent to participate

International ethical guidelines were followed for lymphocytes isolation, after approval by the Ethical Board of the Quaid-i- Azam University, Islamabad, Pakistan (Letter # BEC-FBS-QAU-042/2019). We further confirm that any aspect of the work covered in this manuscript that involved experimental animals has been conducted after approval of experimental protocol (Letter # BEC-FBS-QAU-067/2019) and animal usage (Letter # BEC-FBS-QAU-089/2019) from the Institutional Animal Ethics Committee.

Contributions

We hereby confirm that all authors have made considerable contribution to the work and approved the final version of publication. IH conceptualized and designed the study, supervised execution of experiments, critically revised the manuscript and approved the final version of this manuscript. STBK performed experiments, analyzed and interpreted the data, wrote and revised the manuscript. IN contributed in data curation, validation and critical revision of the final version of the manuscript. HN contributed in the conduction of experiments and acquisition of the data. HF contributed in study design and data validation. ASF contributed in data validation and critical revision of the revised version of the manuscript.

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