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ORIGINAL ARTICLE

Antimicrobial, anti-inflammatory and antioxidant activities of natural organic matter extracted from cretaceous shales in district Nowshera-Pakistan

Fazli Khuda [a,*], Madiha Anjum [a], Suleman Khan [b], Hamayun Khan [a], Muhammad Umar Khayam Sahibzada [c], Ameer Khusro [d], Asif Jan [a], Naveed Ullah [e], Yasar Shah [f], Zakiullah [a], Muhammad Abbas [f], Tayyaba Iftikhar [f], Abubakr M. Idris [g,h], Mayeen Uddin Khandaker [i], Talha Bin Emran [j]

[a] *Department of Pharmacy, University of Peshawar, Peshawar, Pakistan*
[b] *Department of Geology, University of Peshawar, Peshawar, Pakistan*
[c] *Department of Pharmacy, Sarhad University of Science and Information Technology, Peshawar, Pakistan*
[d] *Research Department of Plant Biology and Biotechnology, Loyola College, Chennai 34, Tamil Nadu, India*
[e] *Department of Pharmacy, University of Swabi, Swabi, Pakistan*
[f] *Department of Pharmacy, Abdul Wali Khan University, Mardan, Pakistan*
[g] *Department of Chemistry, College of Science, King Khalid University, Abha 61421, Saudi Arabia*
[h] *Research Center for Advanced Materials Science (RCAMS), King Khalid University, Abha 61421, Saudi Arabia*
[i] *Centre for Applied Physics and Radiation Technologies, School of Engineering and Technology, Sunway University, Bandar Sunway, 47500, Selangor, Malaysia*
[j] *Department of Pharmacy, BGC Trust University Bangladesh, Chittagong 4381, Bangladesh*

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Cretaceous shales;
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Abstract Traditionally, Natural Organic Matter (NOM) derived from cretaceous rocks has been used for treatment of various ailments such as diabetes, inflammation and skin infections. This study evaluated the antimicrobial, antioxidant and anti-inflammatory activities of natural organic matter obtained from cretaceous shales. The shales were collected from Lumshiwal formation; located north of the main Kala Chitta range in district Nowshera-Pakistan. Isolation was done by sonicating crushed rock sample with chloroform, methanol and acetone (70: 15: 15 *v*/*v*, respectively). Antibacterial and antifungal activity of sample was determined by *agar well diffusion* and *Agar slanting methods*, respectively. In vitro anti-inflammatory activity was performed using

* Corresponding author.
E-mail address: fazlikhuda@uop.edu.pk (F. Khuda).
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cyclooxygenase-2 and 5-lipoxygenase enzymes. Antioxidant activity was assessed for scavenging of *DPPH, superoxide anions, hydroxyl radicals,* and *hydrogen peroxide*. In vivo anti-inflammatory activity was performed using "*Carrageenan-induced paw edema model*". The sample showed significant antibacterial activity against *Salmonella typhi*, *Pseudomonas aeruginosa* and *Escherichia coli* with MIC values 0.82, 0.87 and 0.79 mg/ml, respectively. Considerable inhibition was observed against *Bacillus subtilis* (MIC; 0.93 mg/ml) and *Staphylococcus aureus* (MIC; 1.12 mg/ml) when compared with Imipenem as a standard. Moreover, the sample displayed significant antifungal activity against *Alternaria alternata* and *Fusarium solani* with MIC values of 0.60 and 0.68 mg/ml, respectively. Both *COX-2* (IC_{50} 31.34 µg/ml) and *5-LOX* (IC_{50} 38.45 µg/ml) enzymes were inhibited by NOM in a concentration-dependent manner. In addition, the NOM exhibited significant free radical scavenging, especially against DPPH and superoxide anions; and a moderate effect on hydroxyl and hydrogen peroxide scavenging. In vivo anti-inflammatory activity revealed that the edema volume was significantly ($P < 0.001$) decreased at all doses when compared with control and maximum activity (33, 47 and 54% at 50, 100 and 200 mg/kg dose, respectively) was observed at fifth hr of treatment. Likewise, the inhibition capacity was increased with dose. The present findings showed that cretaceous shales may contain a variety of medicinal agents that are traditionally believed to possess properties useful in the treatment of various ailments particularly skin and inflammatory disorders. Therefore, these shales could be a new source for activity-guided isolation of antimicrobial, antioxidant, and anti-inflammatory agents.

1. Introduction

Globally, many researchers are interested in the discovery of more effective drugs from various sources. Natural sources including plants, colonizing bacteria, fungi and microalgae have been exploited for the discovery of new compounds (Ahmed et al., 2020). It has been documented that most of the marine organisms produce antimicrobial compounds as natural defence against the invading pathogens (Maria et al., 2021). Compounds isolated from different marine organisms have shown promising activity against human pathogens (Weiwei et al., 2019). However, irrational use of antibiotics has resulted in a significant increase in the number of drug-resistant microbes (Thangaraju and Venkatesan, 2019). Similarly, nonsteroidal anti-inflammatory drugs (NSAIDs) are effective in treating inflammation and pyrexia; however, they are associated with a number of side effects, particularly peptic ulcers and other GIT issues (Lawrence and Julio, 2021). Contrary to that, the NOM was extracted and subsequently dissolved for various activities using solvent systems having no associated health hazards. The complete process can be referred to the methodology section. To address these challenges, there is an urgent need to investigate new natural sources for the discovery of lead compounds with improved efficacy and minimum side effects.

Black shales are organic rich sedimentary rocks formed as a result of ocean anoxia or higher biological productivity within ocean (Fig. 1). The depositions of such shales are closely associated with periods of global warming in geological record (Naohiko et al., 2015). The enhanced hydrological cycles are closely associated with such global warming periods that may have resulted in flushing of different continental pathogens/microbes into ocean by streams and rivers. The activation of indigenous oceanic pathogens, as well as the influx of continental pathogens, could have increased the immunity of marine zooplankton and phytoplankton via production of different chemicals. These plankton are the sources of organic matter which has been preserved within the black shale (Chaoyong et al., 2021). The composition of NOM depends upon the type of precursor materials deposited during cretaceous period.

Humic substances or NOM are biologically active organic macromolecules of vegetative, animal or microbiological origin that are widely spread in nature. They are predominantly found in soil, water, peat, humus, and various shales. Based on their solubility, humic substances are divided into three components: humic and fulvic acids (alkali soluble), and humin (insoluble residue). Overall, these substances contain major functional groups including carboxylic, carbonyl, phenolic, amides, amines and aliphatic moieties (Table 1) (Laurynas et al., 2021; Mao et al., 2017). Humic acid

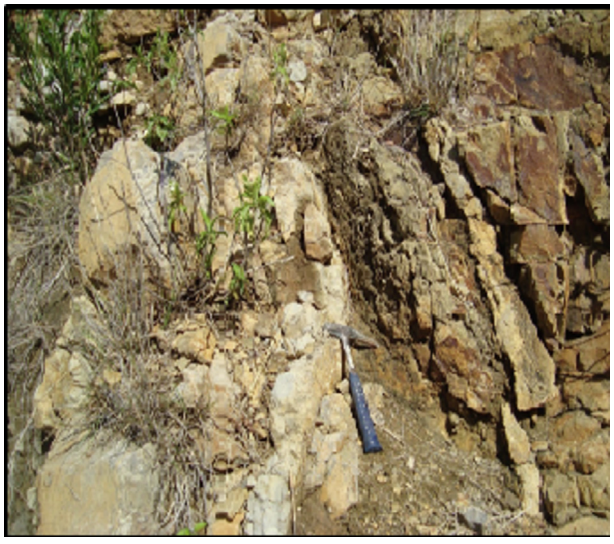


Fig.1 Lumshiwal rock formation located north of the main Kala Chitta range in district Nowshera-Pakistan.

Table 1 Composition of Natural Organic Matter.

Organic matter	Fractions	Identified Compounds	Chemistry
NOM	Soluble NOM	Humic acid	-ArOH and –COOH.
		Fulvic acid	-ArOH, =Ar = and -ArCOOH.
	Insoluble NOM	Humin	Biomacromolecules with variety of functional groups like –CHO, -C = O,–COOH, –NH2, -RH, –CH3O etc.

NOM Natural organic matter; ArOH Phenol; –COOH Carboxylic acid; -=Ar = Conjugated aryls; -ArCOOH Aromatic carboxylic acid; –CHO Aldehydes; -C = O Carbonyls; $–NH_2$ Amines; -RH Phenyl; $–CH_3O$ Methoxy.

(Fig. 2A) is a major component of humic substances, dark brown in color, soluble at alkaline pH and have moderate molecular weight carboxylic and phenol groups (Maguey-Gonzalez et al., 2018). Fulvic acid (Fig. 2B) is yellow in color, soluble in a number of solvents and have low molecular weight carboxylic groups. On the other hand, the insoluble NOM is thought to be a biomacromolecules composed of variety of organic functional groups including aldehyde, ketones, carboxyl, amide, aromatic, alkyl, methoxy and amines (Agrawal and Sharma, 2018). The studied components responsible for medicinal activities of soluble NOM are humic acid and fulvic acid (Winkler and Ghosh, 2018; Robert et al., 2021; Ravin et al., 2017).

Humic acid have been used since ancient times in Indian Ayurvedic medicines for the treatment of diabetes, inflammation and cognitive disorders (Igor et al., 2009; Goel et al., 1990). Humic substances in aquatic systems and water sediments have been linked with the efficacy of balneotherapy and hydrotherapy (Senesi et al., 1991; Gadzhieva et al., 1991). In addition, these substances have been investigated for anticancer, antimicrobial, antiviral, antimutagenic, and wound healing properties (Metin et al., 2018; Maguey-Gonzalez et al., 2018; Winkler and Ghosh, 2018). Some studies have shown that humic acid can inhibit virus replication (Neyts et al., 1992). Their phenolic groups act as electron donating agents, scavenge free radicals, and inhibit chain initiation reaction in autoxidation. They can form micelles and could act as solubilizing agents for a variety of cosmetic and pharmaceutical agents (De-Melo et al., 2016). These properties are useful in formulating cosmetic preparations.

Humic substances from different sources have been investigated for a number of biological activities however; there is still a dearth of scientific validation of the traditional claims of preserved NOM, particularly of cretaceous origin. Thus, the present study was designed to assess the antimicrobial, anti-inflammatory and antioxidant effects of NOM with a view of finding alternate sources of antimicrobial, anti-inflammatory, and antioxidant agents.

2. Material and methods

2.1. Chemicals

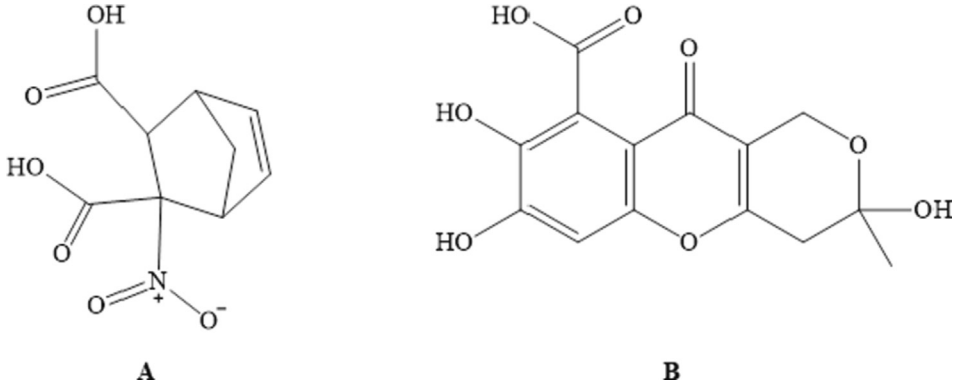
Miconazole, Imipenem and indomethacin were donated by Ferozsons Laboratories Limited, Nowshera, KPK, Pakistan. 1,1-diphenyl 2-picryl hydrazyl, nitro blue tetrazolium, phenazine methosulfate-nicotinamide adenine dinucleotide, 1,10-phenanthroline, cyclooxygenase and 5-lipoxygenase enzymes were purchased from Sigma-Aldrich, Germany. Merck (Darmstadt, Germany) provided hydrochloric acid, hydrofluoric acid, phosphate buffer, methanol and other solvents.

2.2. Collection of cretaceous shale

The cretaceous shales were collected from Kala Chitta Range along Tora Stana in District Nowshera, Khyber Pakhtunkhwa, Pakistan (Lat. 33° 72′ 30″N: Long. 71° 96′ 65″). The sample was identified by Dr Suleiman Khan, Department of Geology, University of Peshawar, Pakistan. The samples were first washed with methanol, followed by crushing and subsequently stored in polythene bags for further processing. The sample was crushed in "Manual Processing Lab" at National Centre of Excellence (NCE), Geology, University of Peshawar.

2.3. Extraction of soluble organic matter

The powdered sample (25 g) was treated with hydrochloric acid, to remove carbonaceous matters; followed by repeated rinsing with distilled water. For removal of silicates, the resi-



A B

Fig. 2 Chemical structure of (A) Humic acid and (B) Fulvic acid.

due was stirred with a mixture of hydrofluoric and hydrochloric acids (1:1 *v*/*v*) and again washed with distilled water until the rinsing become neutral. To remove heavy minerals, the soft residue was stirred with zinc bromide and distilled water. Finally, flakes of organic matter were sonicated in a mixture of chloroform, methanol and acetone (70: 15:15 v/v) and kept for 3 days for maximum extraction. It was then evaporated under vacuum using rotary evaporator. The process was done at Applied Pharmaceutical Lab, Department of Pharmacy, University of Peshawar (Agrawal and Sharma, 2018).

2.4. Isolation of humic substances

Humic substances were isolated using the following methods.

2.4.1. Humic acid

50 g of powdered sediment was treated with sodium hydroxide solution (0.4 L; 0.1 M) in distilled water. The extraction was carried out for 24 h with occasional stirring. After 24 h, the supernatant was collected and centrifuged at 10,000 × g for 15 min. The supernatant was then acidified up to pH 1 with hydrochloric acid solution (4.0 M) and left overnight to precipitate the humic acid. The precipitate was then obtained by centrifugation at 10,000 × g for 15 min (Laurynas et al., 2021).

2.4.2. Fulvic acid

The supernatant (Crude fulvic acid) was filtered through 0.2 µm cellulose nitrate membrane filter and neutralized with sodium hydroxide solution (0.1 M; pH 4 to 7). Solutions were left overnight to precipitate fulvic acid. Precipitates of fulvic acid was then obtained by centrifugation at 6000 × g for 15 min (Laurynas et al., 2021).

2.4.3. Characterization of humic and fulvic acid

2.4.3.1. Physical characterization. Both the acids were checked for physical properties including colour, odour, and solubility.

2.4.3.2. pH determination. The pH of the isolated acids were determined for 2% solution with the help of Mi 150 pH meter at room temperature.

2.4.3.3. UV–Vis spectral analysis. UV-visible spectroscopy was carried out for both the acids and individual spectrums were recorded from 200 to 800 nm on Perkin Elmer Lambda 35 spectrophotometer.

2.5. In vitro studies

2.5.1. Bacterial strains

The following strains of bacteria were used; *Bacillus subtilisin* (ATCC 6633), *Staphylococcus aureus* (ATCC 25924), *Salmonella typhi* (19430), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922). The bacterial suspension was standardized to a turbidity equivalent to 0.5 on the McFarland scale (CLSI, 2012).

2.5.2. Agar-well diffusion method

Microbial cultures were prepared using a validated method (Fazli et al., 2021). Different strains were added to Muller Hinton Agar (MHA) and poured into sterile petri dishes. Well (6 mm) were made using a sterile borer into the agar plate for both control and test samples. 3 mg of NOM was dissolved in DMSO (1 ml; 3%) and about 100 µl of it was transferred into each well. The petri dishes were then incubated for 48 h (37℃) and finally, zone of inhibition was measured (n = 3). Imipenem and DMSO were used as positive and negative control, respectively.

2.5.3. Fungal strains

Fungal strains include *Aspergillus niger* (ATCC 10568), *Alternaria alternata* (ATCC 96255), *Fusarium solani* (ATCC 11213) and *Aspergillus terreus* (ATCC 20445).

2.5.4. Agar slanting method

The selected fungal strains were grown on Sabouraud dextrose agar (SDA) and subsequently incubated for 24 h at 37℃ 4 ml of SDA along with fungal inoculum was poured into sterilized test tubes. Test sample was prepared by dissolving 23 mg in 1 ml of DMSO (3%) and 67.5 µl of it was added into each test tube. The cultured media was solidified in tilting position and then incubated for seven days at 37℃. Miconazole was used as positive control. Finally, the linear growth inhibition was measured and percent inhibition was calculated using Eq. (1). (Fazli et al., 2021)

$$\% \text{ inhibition} = 100 - \frac{\text{Linear growth (test)}}{\text{Linear growth (control)}} \times 100 \qquad (1)$$

2.5.5. Minimum inhibitory concentration (MIC)

The MIC was determined by the broth microdilution method, using 96 well microtiter plates (CLSI, 2012). Initially, NOM was dissolved in DMO and serially diluted with water (sterile) to get various dilutions (i.e. 0.1–30 mg/ml), in microtiter plates. To each well, susceptible strains of growing cultures (1.5108 cfu/ml) were added and subsequently incubated at 37℃ for 72 and 24 hr for fungi and bacteria, respectively. Tetrazolium salt was used as an indicator. It was added to each well and the appearance of violet color in culture media indicated the growth of microorganisms. Imipenem and miconazole were used as positive, while DMSO was used as a negative control.

2.5.6. Cyclooxygenase (COX-2) assay

The method of Olomola et al. was used for the determination of in vitro enzyme inhibition activity (Olomola et al., 2020). For 10 min, the enzyme (300 U/ml) and co-factor (50 µl) were preincubated on ice. In addition 50 µl of co-factor solution (1 mM Hematin, 0.9 mM glutathione and 0.24 mM N,N,N, N-tetramethyl-pphenylenediamine dihydrochloride in 0.1 M Tris HCl buffer, pH 8.0) was mixed with the enzyme solution. Subsequently, the enzyme solution and NOM (25–1000 µg/ml) were mixed and allowed to stand for 5 min at 25℃. In order to initiate the reaction, arachidonic acid (20 ml, 30 mM) was added to this mixture and incubated for 4 to 5 min. Finally, maximum absorbance was determined at 570 nm.

2.5.7. 5-lipoxygenase (5-LOX) assay

Initially, solutions of montelukast, NOM (25–1000 µg/ml), phosphate buffer (50 mM, pH 6.3) and *5-LOX* enzyme (10,000 U/ml) were prepared. Test samples were dissolved in

buffer solution (250 µl) and the enzyme solution (250 µl) was added to it before incubation at 25℃ for 5 min. In addition, the enzyme solutions and linoleic acid (0.6 mM, 1 ml) were mixed and maximum absorbance (234 nm) was determined with a UV–vis spectrophotometer (Okur et al., 2021). Percent inhibition values were determined using Eq. (2).

$$\text{Percent Inhibition} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} \times 100 \quad (2)$$

2.5.8. Antioxidant activity

2.5.8.1. DPPH assay. The antioxidant activity of NOM was determined with 1,1-diphenyl 2-picryl hydrazyl radical (DPPH) spectrophotometrically at 517 nm. The DPPH was dissolved in methanol (100 ml) to get 0.01 mM solution. Stock solution of NOM (1 mg/ml) was prepared in methanol. Using suitable dilution, working solutions of the sample (25, 100, 200 and 500 µg/mL) were prepared from it. From each concentration, 100 µl was added to DPPH solution (3 ml). The absorbance was measured after 15 min of incubation at 23℃. In this assay, ascorbic acid was used as a standard drug (Ahmad et al., 2020). The percentage DPPH radical scavenging potential was measured using Eq. (3).

$$\%\ \text{Radical scavenging potential} = \frac{C_{Abs} - S_{Abs}}{C_{Abs}} \times 100 \quad (3)$$

Where C_{Abs} and S_{Abs} are the absorbance of the control and test samples/standard, respectively.

2.5.8.2. Superoxide anion radical scavenging activity. This activity was measured by the reduction of nitro blue tetrazolium (NBT) based on a previous method. Initially, the superoxide radicals were generated in a phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system. In this assay the radicals were generated in sodium phosphate buffer (3 ml, 100 mM, pH 7.4) containing NBT (1 ml, 150 µM), NADH (1 ml, 468 µM) solutions, along with various concentrations of NOM (25–1000 µg/ml) in water. The reaction was initiated by the addition of PMS (1 ml, 60 µM) to the mixture. After incubation (5 min, 25℃) the maximum absorbance was measured at 562 nm, against the corresponding blank solution. Ascorbic acid was used as standard (Sourav et al., 2011).

2.5.8.3. Hydroxyl scavenging activity. This activity was determined with Fenton reaction. Reaction mixture contained $FeCl_2$ (60 µl, 1.0 mM), H_2O_2 (150 µl, 0.17 M), 1,10-phenanthroline (90 µl, 1 mM), phosphate buffer (2.4 ml, 0.2 M) and 1.5 ml of NOM at various concentrations (25–1000 µg/ml). The reaction was started by the addition of H_2O_2. After incubation (25℃, 5 min), the maximum absorbance was measured at 560 nm (Yupiao et al., 2018).

2.5.8.4. Hydrogen peroxide scavenging. An aliquot of H_2O_2 (50 mM) and various concentration of NOM (25–1000 µg/ml) were mixed (1:1 *v*/*v*) and subsequently incubated for 25 min at 25℃. Following incubation, 80 µl of H_2O_2 and NOM solution was mixed with methanol (10 µl) and FOX reagent (0.9 ml) was added to it (FOX reagent was prepared by mixing BHT (9 volumes, 4.4 mM) in methanol with xylenol orange (1 vol, 1 mM) and ammonium ferrous sulfate (2.56 mM) in 0.25 M H_2SO_4). The reaction mixture was vortexed (25 min) before incubation at 25℃. The absorbance of the complex (ferric-xylenol orange) was measured at 560 nm. Sodium pyruvate was used as standard (Shumaila et al., 2013).

2.6. In vivo studies: Anti-inflammatory activity

2.6.1. Animals

Healthy BALB/c mice (25–30 g) of either sex were used for the *in vivo* anti-inflammatory activity. Standard protocol was adopted for conducting the experiments. The experiment was approved by the "*Ethical Committee of the Department of Pharmacy, University of Peshawar*" (Application No. 03/EC-18/Phar).

2.6.2. Carrageenan-induced paw edema

The *in vivo* anti-inflammatory activity was determined using carrageenan-induced paw edema model (Lingadurai et al., 2007). The animals were divided into five groups (n = 5). They were treated with NOM (50, 100 and 200 mg/kg, i.p), indomethacin (10 mg/kg, i.p), and normal saline. Paw edema was induced by injecting carrageenan solution (0.05 ml; 1%), 1 hr after the administration of NOM, into the left hind paw's subplantar region. The standard and control groups received indomethacin and normal saline, respectively. The linear paw circumference was measured before and 1, 2, 3, 4 and 5 h after the injection of carrageenan, using a digital plethysmometer (model 7150, Ugo Basile, Italy). Percent inhibition was measured using Eq. (4).

$$\%Inhibition = \left(\frac{C - T}{C}\right) \times 100 \quad (4)$$

where T and C represent the increase in paw volume of the test and control treatment groups, respectively.

2.7. Statistical analysis

Statistical analysis was conducted using One-way ANOVA along with Dunnet post hoc test (GraphPad Software Inc., San Diego, California, USA); data was presented as mean ± SEM for each group of five animals.

3. Results and discussion

3.1. Isolation of humic substances

Two fractions of NOM were isolated from organic rich cretaceous shale employing repeated sonication with polar solvents followed by subsequent treatment with acids (HCL/HF). The yield of isolated soluble NOM was < 0.1 %. Furthermore, individual compounds (humic and fulvic acids) thought to be responsible for medicinal activities, were isolated using pH derived precipitation methods and identification was carried out by physical characterization.

3.2. Physical characterization

In present study, soluble NOM appears as yellowish brown, while insoluble NOM as black powder. Fulvic and humic acid

was golden-yellow and brown in color, respectively (Fig. S1). Fulvic acid exhibited muddy or candy like odour while humic acid possess no odour (Table S1).

3.2.1. pH determination

The pH of fulvic and humic acid solution (2%) was 3.2 and 3.8, respectively.

3.2.2. UV–Vis spectral analysis

UV–Vis spectrums of fulvic and humic acid was in the range of 200 to 600 nm and 200 to 550 nm, respectively (Fig. S2). Both the acids exhibit wide uncharacteristic spectra consistent with earlier reported studies conducted on structural elucidation of natural organic matter (Kyoichi, 1987).

3.3. In vitro studies

3.3.1. Agar-well diffusion method

The cretaceous period was the warmest period in earth history. During that period, the temperature raised three times than the current global temperature (Ohkouchi et al., 2015). As a result, the polar glaciers melted and all water was added to oceans. The high global temperature and water on continents and ocean might have activated different strains of pathogens because they are sensitive to high temperature and water. The diverse pathogens from continents were shredded into oceans which might have further added to the diversity of oceanic pathogens. In response, the immunity of phytoplankton and zooplankton might have enhanced by producing different chemicals. The same algae and cyanobacteria are now preserved in rock record. Therefore, this study was designed to preliminary investigate the antimicrobial potential of NOM which may lead to the discovery of new lead compounds.

In present study, the NOM was tested for antimicrobial activity against some well-known pathogens, including *P. aeruginosa, S. typhi*, *S. aureus, B. subtilis* and *E. coli*. Literature review showed that typhoid fever, skin and other frequent infections are caused by these bacteria (Eng et al., 2015). *E. coli* is the most common facultative anaerobe in humans, causing illnesses such as gastroenteritis, urinary tract infection, meningitis and peritonitis (Arwyn-Jones and Surjo, 2019).

NOM exhibited significant activity against *S. typhi*, *P. aeruginosa, E. coli* and *B. subtilis* with MIC values 0.82, 0.87, 0.79 and 0.93 mg/ml, respectively. In addition, it showed considerable activity against *S. aureus* with MIC values 1.12 mg/ml (Table 2).

The antibacterial activity shown by NOM may involve several mechanisms including, but not limited to, pore formation, nucleic acid synthesis, DNA gyrase inhibition, and change in physiochemical properties of the plasma membrane (JJose and Cesar, 2016). It has been previously reported that humic and fulvic acids possess significant antibacterial activity particularly against methicillin-sensitive *S. aureus*, *S. thyphi*, *MRSA*, *E. coli*, and *P. aeruginosa* (Maguey-Gonzalez et al., 2018; Winkler and Ghosh, 2018). According to literature review, both fulvic and humic acids are the prominent constituents of NOM. Therefore, the antibacterial activity of NOM in this study against the mentioned pathogens may be partially due to these compounds.

3.3.2. Agar slanting method

Despite the fact that there are few studies in the literature regarding the antifungal activity of NOM. Here, it proved to be active against all the tested fungi especially against *A. alternata* and *F. solani* with MIC values 0.60 and 0.68 mg/ml, respectively, whereas it showed considerable activity against *A. niger* and *A. terreus* with MIC values 0.82 and 1.10 mg/ml, respectively (Table 3). Fusarium and Alternaria are well-known plant pathogens responsible for significant productivity losses in agricultural crops globally. These fungi causes early blight and wilt of tomato (Haytham et al., 2021). It has been documented that synthetic fungicides are extensively used to control plant pathogens; however, in addition to its toxicity to consumers they are considered the most lethal pollutants for the environment. Contrary to that, natural pesticides are environment friendly with minimal or no health hazards or pollution (Asmaa et al., 2020). Based on our results NOM may be a potential source for developing an eco-friendly phytofungicides.

3.3.3. Cyclooxygenase and lipoxygenase assays

Both *COX-2* and *5-LOX* are considered as pro-inflammatory enzymes, involved in arachidonic acid metabolism (Violette and Ebtisam, 2018). They are also involved in the production of eicosanoids, including thromboxane, leukotrienes and prostaglandins that primarily cause inflammation. Therefore, these enzymes are the rate-limiting factors in the production of mediators of inflammation and commonly used for screening anti-inflammatory drugs. It is pertinent to mention that co-inhibition of *COX/5-LOX* potentially reduce side effects preferably on the GIT and cardiovascular system, while retaining the activity of *COX-1/2* inhibitors (Martel-Pelletier et al., 2003).

Table 2 Antibacterial activity of NOM against selected bacterial strains.

Test microorganisms	NOM			Standard	
	Inhibition zone (mm)	MIC	% Inhibition	Inhibition zone	MIC
Pseudomonas aeruginosa	16	0.87	48	33	0.0023
Salmonella typhi	17	0.82	49	35	0.0012
Staphylococcus aureus	9	1.12	32	28	0.0011
Bacillus subtilis	11	0.93	42	26	0.0014
Escherichia coli	15	0.79	49	32	0.0022

MIC: Minimum inhibitory concentration (mg/ml)
Standard: Imipenem

Table 3 Antifungal activity of NOM against selected fungal strains.

Test microorganisms	NOM		Standard	
	% Inhibition	MIC	% Inhibition	MIC
Fusarium solani	53	0.68	68	0.0017
Alternaria alternata	62	0.60	76	0.0001
Aspergillus niger	38	0.82	69	0.0004
Aspergillus terreus	25	1.10	55	0.0019

Antifungal activity of NOM against selected fungal strains
MIC: Minimum inhibitory concentration (mg/ml)
Standard: Miconazole

Based on its traditional uses, we investigated the anti-inflammatory effect of NOM using in vitro models. In this study, *COX-2* and *5-LOX* enzymes were inhibited by NOM in a dose-dependent manner. The *COX-2* inhibition results showed that NOM had significant activity with IC_{50} value of 31.34 µg/ml, compared to reference drugs Celecoxib (18.23 µg/ml). Concerning *5-LOX* inhibition capacity, the sample showed considerable activity with IC_{50} value of 38.45 µg/ml, compared to Montelukast (25.34 µg/ml) (Fig. 3).

Inflammatory reactions are commonly triggered by higher level of prostaglandins and leukotrienes, which are produced by *5-LOX* and *COX-1/2* enzymes, respectively. Prostaglandins sensitize nociceptive fibres and facilitates the passage of pain stimuli, while leukotrienes stimulates cell adhesion to vascular endothelium (Felipe et al., 2017). Therefore, inhibition of *COX-1/2*, reduce prostaglandin production, which subsequently reduce pain and inflammation. However, inhibition of prostaglandins may lead to activation of *5-LOX* pathway, which in turn raise gastrotoxic leukotrienes. The use of dual inhibitors of *COX-2/5-LOX*, help to reduce these problems (Afshin and Sara, 2011). The results from the current study clearly exhibited that NOM had a high potency against *COX* and *LOX* and thus may be considered in anti-inflammatory drug discovery and development.

3.3.4. Antioxidant activity

According to WHO, nearly 80% of the world population rely on herbal traditional medicines for their primary health care needs and most of these therapies involves the use of herbal extracts (Nisa et al., 2013). It has been documented that our body produces more reactive oxygen species (ROS) than enzymatic antioxidants under stress condition. These includes superoxide anion radical ($O_2^{\cdot-}$), perhydroxyl radical ($HO_2\cdot$) and hydroxyl radical (·OH) as well as some non-free radicals i.e·H_2O_2 (Duduku et al., 2011). In the absence of antioxidants, these free radicals facilitates the development of several diseases which includes but not limited to cancer, liver cirrhosis, diabetes, atherosclerosis, cardiovascular diseases, inflammation, and a variety of neurodegenerative disorders (Nisa et al., 2013; Sajjad et al., 2015). Synthetic antioxidants such as butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) have been widely used in food industry but these agents are reported to be associated with carcinogenesis and liver cirrhosis (Duduku et al., 2011). Therefore, in last few decades the use of natural antioxidants have got much attention.

3.3.4.1. DPPH assay. Several *in vitro* models have been documented for investigating the scavenging activity in various compounds. The DPPH is a stable organic radical commonly used to assess the free radical scavenging potential of phytochemicals and extracts. When the stable DPPH accepts an electron from the antioxidant specie, its violet color is reduced to yellow diphenylpicrylhydrazine radical, which is colorimetrically measured (Sagar and Sing, 2011). In present study, NOM showed significant activity 18, 31, 42, 51, 63 and 72% at concentrations of 25, 50, 100, 250, 500 and 1000 µg/ml, respectively. As shown in Table 4, the sample depicted a dose dependent activity, compared to ascorbic acid, which showed 95% DPPH scavenging at 1000 µg/ml. Traditional medicines have been used since long time for the treatment of a variety of ailments and their scientific validation have led to the development of many novel drugs. Results of the present study suggests that NOM may be considered as a potential source for the discovery of natural antioxidants.

3.3.4.2. Superoxide anion radical scavenging activity. It has been reported that superoxide anions directly or indirectly damage biomolecules during pathological events such as inflammation by forming H_2O_2, singlet oxygen or ^{-}OH. They can also directly initiate lipid peroxidation (Małgorzata and Andrzej, 2016). The superoxide radicals (produced from dis-

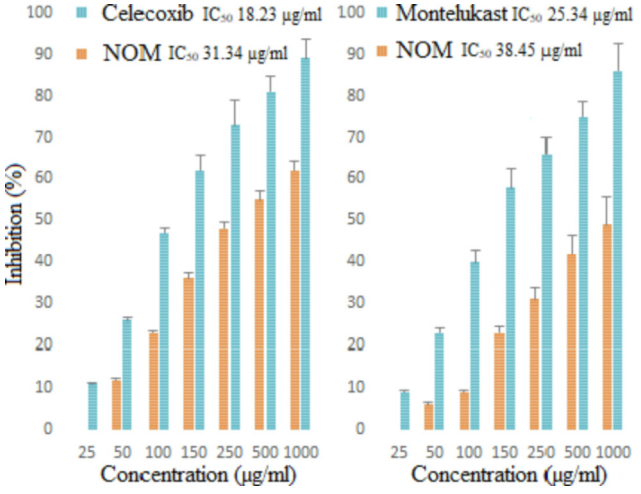


Fig.3 The relative inhibition of COX-2 and 5-LOX enzymes by NOM and standard.

Table 4 DPPH and Superoxide anion radical scavenging activity of NOM and ascorbic acid.

Concentrations (µg/ ml)	DPPH Scavenging activity (%)		Superoxide anion radical scavenging (%)	
	NOM	Ascorbic acid	NOM	Ascorbic acid
25	18.12 ± 0.32	28.22 ± 0.33	11.20 ± 0.20	19.05 ± 0.18
50	31.22 ± 0.55	43.14 ± 0.56	28.10 ± 0.25	39.18 ± 0.45
100	42.12 ± 1.22	55.10 ± 1.21	35.12 ± 0.90	47.16 ± 1.20
250	51.45 ± 1.50	71.25 ± 1.32	44.12 ± 0.78	62.15 ± 1.66
500	63.40 ± 1.65	82.12 ± 1.50	53.23 ± 1.13	74.21 ± 1.34
1000	72.15 ± 1.88	95.18 ± 1.65	68.10 ± 1.0	88.12 ± 1.34

solved oxygen by PMS-NADH coupling) can be quantified by their ability to reduce NBT. The reduction in absorbance at 560 nm caused by NOM shows its potential to quench superoxide radicals in the reaction. As shown in Table 4, the radical scavenging activity of NOM markedly increased with increase in concentration. In this assay, the sample and standard showed 68 and 88% superoxide anion scavenging at 1000 µg/ml, respectively. The results suggest that NOM can be further investigated for the isolation of specific compounds responsible for superoxide radical scavenging effects.

3.3.4.3. Hydroxyl scavenging activity. Hydroxyl radicals are the primary active oxygen species responsible for lipid peroxidation and massive biological injuries. These radicals are generated from the reaction of transition metals with various hydroperoxides. It damages unsaturated fatty acids in membrane, proteins, DNA and other biological molecules (Anju et al., 2019). In this study, NOM depicted concentration dependent activity against hydroxyl radicals produced in the Fenton reaction. As shown in Fig. 4, the sample and standard exhibited 73 and 92% hydroxyl scavenging activity at 1000 µg/ml, respectively.

3.3.4.4. Hydrogen peroxide scavenging. This is a weak oxidizing agent that directly inactivates few enzymes, typically by oxidation of essential thiol (-SH) groups. It has the ability to rapidly cross cell membranes; once inside the cell, it is likely to react with Fe^{2+} and possibly Cu^{2+} ions to form ^{-}OH radicals, which may be the source of many of its toxic effects (Ergul, 2016). NOM showed weak hydrogen peroxide scavenging activity i.e. 40 % at the highest tested dose, compared to standard, which depicted 73% activity at the same dose (Fig. 5).

These *in vitro* tests show that NOM contains a significant amount of natural antioxidants, which may aid in the prevention of a variety of oxidative stresses. However, the compounds responsible for the antioxidant activity are unknown at this time. As a result, additional research is required to isolate and identify the antioxidant compounds present in NOM. Furthermore, the antioxidant activity of NOM *in vivo* must be determined before it can be used in clinical trials.

3.4. In vivo studies: Anti-inflammatory activity

3.4.1. Carrageenan-induced paw edema

The anti-inflammatory activity of NOM against carrageenan-induced paw edema is shown in Table 5. The NOM at all doses significantly ($P < 0.001$) decreased the edema volume against control and maximum activity (33, 47 and 54% at 50, 100 and 200 mg/kg dose, respectively) was observed at fifth hr. of treatment. The standard indomethacin showed maximum inhibition (58%) at fourth hr. of administration.

It is evident from the above results that NOM has significant anti-inflammatory potential by controlling biphasic

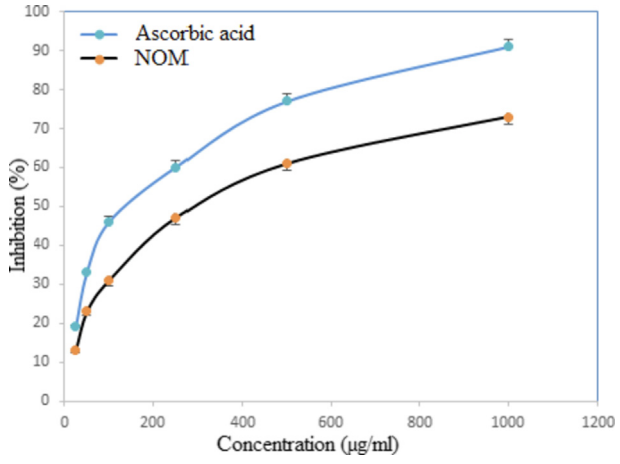


Fig. 4 Hydroxyl scavenging activity of NOM and standard at different concentrations. Each value represents mean ± SD (n = 3).

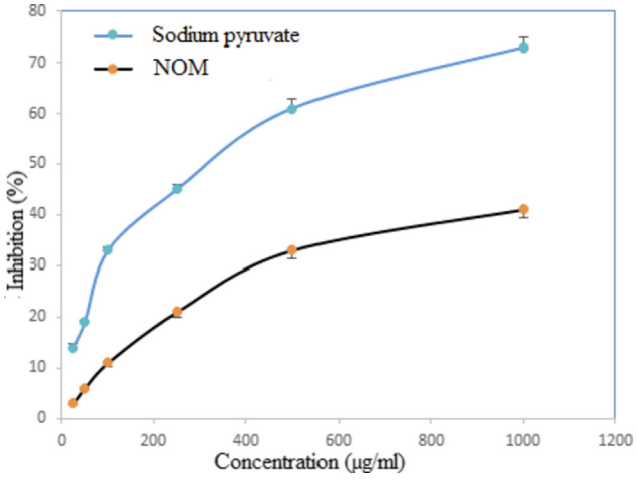


Fig. 5 Hydrogen peroxide scavenging activity of NOM and standard at different concentrations. Each value represents mean ± SD (n = 3).

Table 5 Anti-inflammatory effect of NOM on carrageenan-induced paw edema following intraperitoneal administration in mice.

Groups	Mean change in the hind paw volume (ml)						
	Dose (mg/kg.i.p)	BV	1 h	2 h	3 h	4 h	5 h
Control	–	0.71 ± 0.02	1.19 ± 0.09	1.20 ± 0.08	1.21 ± 0.08	1.19 ± 0.08	1.18 ± 0.07
NOM	50	0.70 ± 0.02	0.97 ± 0.03 (17.31 %)[c]	0.98 ± 0.12 (21.1%)	1.10 ± 0.09 (13.98%)	0.93 ± 0.10[b] (25.99%)	0.80 ± 0.05[b] (33.89%)
NOM	100	0.65 ± 0.02	0.94 ± 0.04 (23.2%)	0.94 ± 0.07 (29%)	0.88 ± 0.07[a] (39%)	0.76 ± 0.04[b] (44%)	0.71 ± 0.01[b] (47%)
NOM	200	0.60 ± 0.04	0.80 ± 0.07 (27%)	0.77 ± 0.03[a] (35%)	0.75 ± 0. 03[a] (43%)	0.69 ± 0.05[b] (48%)	0.57 ± 0.2[b] (54%)
Std	10	0.58 ± 0.2	0.75 ± 0.03[b] (39%)	0.73 ± 0.04[b] (45%)	0.60 ± 0.02[b] (52%)	0.53 ± 0.01[b] (58%)	0.57 ± 0.04[b] (55%)

Values are expressed as mean ± SEM, n = 5, [a]$p \leq 0.01$, [b]$p \leq 0.001$ compared with control, analyzed by one way ANOVA followed by Dunnet's test for multiple comparison; BV: Basal volume; Std; Indomethacin; [c]Values in parentheses indicates the percentage inhibition rate

inflammatory events. Carrageenan-induced inflammation in animals occur in two phases. The early phase (90–180 min) is characterized by the release of histamine, serotonin and similar substance whereas; the later phase (270–360 min) is associated with the release of prostaglandins, lysosome and proteases. In this study, NOM showed significant inhibitory effect particularly in the later phase of inflammation. This suggests that NOM possibly acts by inhibiting the release and/or actions of vasoactive substances (serotonin, histamine) and prostaglandins. The present results corroborate the importance for screening cretaceous shales as a potential source for bioactive compounds. Phytochemical composition of NOM showed the presence of humic substances including fulvic acid and some complex organic matters mainly produced by cyanobacteria (Ohkouchi et al., 2015). Literature review showed that humic substances possess significant anti-inflammatory activity (De-Melo et al,. 2016). In light of the abovementioned facts, this preliminary study could lead to the discovery of novel anti-inflammatory drugs derived from cretaceous shales that can be used for the rapid and effective management of infectious diseases.

4. Conclusion

This study for the first time confirms the antimicrobial as well as antioxidant and anti-inflammatory potential of NOM extracted from cretaceous shales. The NOM showed significant antimicrobial activity against *S. typhi*, *P. aeruginosa*, *E. coli*, *A. alternata* and *F. solani*. In vitro models depicted concentration dependent inhibition of *COX-2* and *5-LOX* enzymes. The NOM revealed good antioxidant activity against common free radicals i.e. *DPPH, superoxide* and *hydroxyl*. It also exhibited significant anti-inflammatory activity particularly at late hrs. of inflammation. These findings suggest that NOM could be a novel source for antimicrobial, anti-inflammatory and antioxidant agents. However, it is important to determine the specific compound(s) responsible for the said activities; and to establish its proper mechanism in order to come to a definite conclusion.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2021.103633.

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