Research article

Biochemical evaluation and molecular docking assessment of the anti-inflammatory potential of *Phyllanthus nivosus* leaf against ulcerative colitis

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A R T I C L E I N F O

A B S T R A C T

Ulcerative colitis (UC) is an inflammation of the colon that can progress to colorectal cancer if left untreated. No medication completely cures UC and natural products are sources of alternative approaches. This study aimed to determine the anti-inflammatory potential of *Phyllanthus nivosus* leaf extract and fractions in a rat model of ulcerative colitis and to identify the active ingredients. UC was induced in rats by intra-rectal infusion of 1ml of 4% acetic acid (AA) in normal saline. AA exposed groups of rats were treated with 100 mg/kg bodyweight of methanol extract, hexane, ethyl-acetate and butanol fractions orally for four days. Another group received the standard drug - Dexamethasone and control rats were given distilled water only. Some biochemical changes were evaluated and the active ingredients were identified using Gas Chromatography-Mass Spectrometry (GC-MS) followed by molecular docking against interleukin-1-beta converting enzyme (Caspase-1), beta-2 adrenergic receptor (ADRB2), cyclooxygenase-2 (COX-2) and tumour necrosis factor-alpha (TNF-α). Exposure of rat colon to acetic acid significantly altered ($p < 0.05$) serum levels of tumour necrosis factor-alpha (TNF-α), interleukin – 6 (IL-6), nitric oxide (NO), lipid peroxidation product (malondialdehyde or MDA), reduced glutathione (GSH); and activities of superoxide dismutase (SOD) and catalase (CAT). These alterations were however restored in the rats treated with *P. nivosus* leaf with the ethyl-acetate fraction displaying the highest ameliorative activity. GC-MS analysis of the ethyl acetate fraction revealed the presence of 40 compounds which when subjected to molecular docking demonstrated varying degrees of binding affinities for the protein targets. Ethyl iso-allocholate demonstrated the highest binding affinity for caspase-1, cholest-22-ene-21-ol, 3,5-dehydro-6- methoxy-, pivalate for ADRB2 and TNF-α; and alpha-cadinol for COX-2. The anti-inflammatory potential of *Phyllanthus nivosus* leaf as a natural remedy and as a source of new drugs against ulcerative colitis is validated.

1. Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) of the same type with Crohn's disease and microscopic colitis [1, 2]. It is a chronic inflammation associated with ulcers of the colorectal mucosa, accompanied by abdominal pain and diarrhoea which may be with mucus, blood, or pus secretion [3]. Although UC has been considered a disease of industrially developed countries [4, 5], the prevalence has risen in developing countries and it's gradually becoming a global disease [6, 7]. While the aetiology remains unknown, ulcerative colitis is suggested to be associated with abnormal immune function, alteration of mucosal sensitivity to intestinal antigens, changes in normal intestinal microflora, genetic alterations and environmental factors [8, 9].

Treatment of UC has been a clinical challenge as classical medicines have not been able to completely cure the disease and the drugs also possibly cause some adverse effects [10]. Treatment begins with 5-aminosalicylic acid which may be followed by regimens like anti-TNF antibodies, steroids or immunosuppressants. However, recurrent inflammatory responses or treatment-resistant disease may be experienced frequently by some patients. This may lead to damage of the

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epithelial surface of the mucosa which may gradually result in uncontrolled epithelial proliferation and eventually colorectal cancer [11]. Herbal medicines and plant products have recently been seen as a promising remedy for UC due to their antioxidant capacity and their regulatory effects on the pro-inflammatory pathway [12].

Phyllanthus nivosus (Bryenia disticha), commonly known as ice cream bush or snow bush is a plant in the family Phyllanthaceae first reported in 1776 [13]. Native to New Caledonia and Vanuatu in the Western Pacific, the plant is richly cultivated in Nigeria as an ornamental plant and as a natural remedy for malaria, fever, headaches, toothaches and tooth infections [14, 15]. Amadi and colleagues (2017) reported the activity of aqueous and ethanol extracts of the leaf against *Streptococcus mutans* isolated from dental caries patients [16]. Onyegbule and colleagues (2014) reported the analgesic, anti-inflammatory and antimicrobial activity of the plant extracts [17]; and Johnson and colleagues (2020) reported its antimalarial activity [18]. This study aimed to evaluate the anti-inflammatory activity of various fractions of *Phyllanthus nivosus* in a rat model of ulcerative colitis and to identify the active ingredients.

2. Materials and methods

2.1. Plant collection

Phyllanthus nivosus leaf was obtained from various locations of Jos North local government area of Plateau state. The authentication was carried out at the Department of Plant Science and Technology, University of Jos, Jos, Nigeria, where a voucher specimen with the number: UJ/PCG/HSP/13E01 was deposited.

2.2. Preparation of extract and fractions

Shade dried and finely powdered *Phyllanthus nivosus* leaf (300 g) was macerated with 1200 mL of 70% methanol for 24 hours at room temperature. The mixture was filtered and the extract air-dried and stored at room temperature in an airtight container. The methanol extract was subjected to solvent partitioning using hexane, ethyl acetate and butanol. The fractions obtained were dried and stored at 4 °C.

2.3. Experimental animals

Twenty-eight Wistar Albino rats of both sexes weighing 180 ± 50 g were obtained from the animal house of the University of Jos. They were acclimatized and maintained in a well-ventilated cage under standard laboratory conditions for 7 days prior to the start of the experiment. The study was conducted with the approval of the University of Jos Animal Ethics Committee with the approval number: UJ/FPS/F17-00379.

2.4. Experimental design

The number of animals per group was calculated using the resource equation approach as described by Arifin and Zahiruddin, 2017 [19]. Based on this calculation, a minimum of 3 and maximum of 4 animals were required for this study. Hence the rats were divided into seven

groups of 4 rats each (n = 4). Ulcerative colitis was induced as described by Millar et al., 1996 [20]. Groups 2 to 7 rats were given 1ml of 4% acetic acid in normal saline intra-rectally using a lubricated catheter and light ether anaesthesia. Group 1 rats were given distilled water only, through the same procedure (Table 1). The instillation site was about 6 cm from the anal verge into the rectum. Before removing the catheter, 2ml air was injected into the rectum to allow the acetic acid (or distilled water) to spread in the colon. The animals were maintained in a Trendelenburg position for 30 seconds to prevent leakage. Intraperitoneal administration of 100 mg/kg body weight extract/fractions (chosen based on previous studies [17]) and 1mg/70kg standard drug (Table 1) started 4 hours after the exposure and continued for four days. Thereafter, the animals were sacrificed under ether anaesthesia. Serum and colon samples were obtained and stored at -20 °C until required for the determination of biochemical parameters.

2.5. Determination of biochemical parameters

Superoxide dismutase (SOD) activity was determined according to Madesh and Balasubramanian (1998) [21], catalase activity according to Sinha (1972) [22], reduced glutathione (GSH) according to Moron *et al.*, (1979) [23], lipid peroxidation (malondialdehyde or MDA) was measured as described by Balasubramanian *et al.*, (1988) [24] and Nitric oxide (NO) level by the Green *et al.*, 1982 method [25]. Tumour necrosis factor (TNF)-α and interleukin (IL)-1b were determined using enzyme-linked immunosorbent assay (ELISA) kits from Biolegend, San Francisco, California, USA.

2.6. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analysis of the ethyl acetate fraction was performed using Shimadzu GC-MS - QP2010 PLUS as previously described [18]. The equipment was set at the following conditions: Injector temperature - 250 °C, oven temperature - 60 °C, ion source temperature - 200 °C, interface temperature - 250 °C, pressure - 100.2 KPa, column flow - 1.61 ml/min, purge flow 5.6 ml/min and total flow time - 39.4 ml/min. Manual injection of the sample was done at a split ratio of 20:0 and the total running time was 11 min. The National Institute of Standard and Technology (NIST) library database was used in the interpretation of the mass spectrum.

2.7. Molecular docking analysis

The chemical compounds obtained from the results of GCMS analysis were subjected to molecular docking with some anti-inflammatory protein targets. These molecular targets include interleukin-1-beta converting enzyme (Caspase-1), beta-2 adrenergic receptor (ADRB2), cyclooxygenase-2 (COX-2) and tumour necrosis factor-alpha (TNF-α) with PDB ID: 1ICE, 3NYA, 5W58 and 2AZ5. 3D Structures of the molecules were obtained from protein databank (www.rcsb.org). The existing ligands and water molecules were removed and hydrogen molecules were added. The SDF (structure-data file) structures of the compounds and standard ligands i.e. pralnacasan, alprenolol, rofecoxib and

Table 1. Grouping of experimental rats.

Group No	Group Name	Exposure (Intra-rectal)	Treatment (Intraperitoneal)
1	Normal control	Distilled water	Distilled water
2	Colitis control	Acetic acid	Distilled water
3	Methanol	Acetic acid	Methanol extract
4	Hexane	Acetic acid	Hexane fraction
5	Ethyl acetate	Acetic acid	Ethyl acetate fraction
6	Butanol	Acetic acid	Butanol fraction
7	Dexamethasone	Acetic acid	Dexamethasone

thalidomide respectively were obtained from the PubChem database. The ligands were docked to the protein targets and the binding affinities were determined using Autodock Vina from PyRX [26]. Molecular interactions between proteins and ligands were viewed with Chimera 1.14 and discovery studio 2020.

2.8. Statistical analysis

Statistical analysis was performed by one-way ANOVA using IBM SPSS 23.0 (SPSS Inc., Chicago, IL, USA) and the results were expressed as mean $\pm$ SD. Group differences were determined by Duncan's multiple comparison test ($p < 0.05$).

3. Results

3.1. Serum biochemical parameters of oxidative stress

Intra-rectal administration of acetic acid significantly reduced ($p < 0.05$) serum superoxide dismutase activity, catalase activity and glutathione concentration (Figure 1); and significantly increased serum levels of malondialdehyde and nitric oxide (Figure 2). Treatment with *P. nivosus* leaf extracts and standard drug significantly changed the altered values with the ethyl acetate fraction displaying the highest ameliorative activity among the extracts as shown in both figures.

3.2. Serum cytokine levels

Levels of interleukin-6 and tumour necrosis factor - α significantly increased ($p < 0.05$) in the serum of rats induced with ulcerative colitis.

All the extracts and the standard drug caused significantly reduced levels of interleukin-6 when compared with the colitis group. With the exception of butanol fraction, the extracts and standard drug also significantly reduced the levels of TNF – α. The ethyl acetate fraction displayed the highest ameliorative activity as there was no significant difference in the cytokine levels of ethyl acetate treated groups compared with the values from normal control groups (Figure 3).

3.3. GC-MS analysis of ethyl acetate fraction of P. nivosus leaf extract

GC-MS analysis of the ethyl acetate fraction revealed the presence of 40 compounds from 43 chromatogram peaks. Peaks 26, 31 and 35 represent 1-nonadecene while peaks 41 and 45 represent 9-hexacosene. The compounds detected comprise some terpenes (beta -pinene, alpha-phellandrene, D-limonene, beta-caryophyllene, phytol, alpha-cadinol and isoledene) and different classes of hydrocarbons, alcohols, steroidal compounds, fatty acids and their derivatives (Figure 4 and Table 2).

3.4. Molecular docking analysis

P. nivosus leaf compounds demonstrated varying degrees of binding affinities for the protein targets as revealed by the change in Gibb's free energy (ΔG) shown in Table 3. The binding affinities of cholest-22-ene-21-ol, 3,5-dehydro-6- methoxy-, pivalate (-8.2 Kcal/mol), ethyl iso-allocholate (-7.5 Kcal/mol), isoledene (-7.0 Kcal/mol), alpha-cadinol (-7.0 Kcal/mol) and nine other compounds including alpha-phellandrene (-6.3) for ADRB2 were found to be higher than that of the standard ligand alprenolol (-7.2 Kcal/mol). Alpha-cadinol, ethyl iso-allocholate, isoledene and 1,4,7,-cycloundecatriene, 1,5,9,9-tetramethyl-
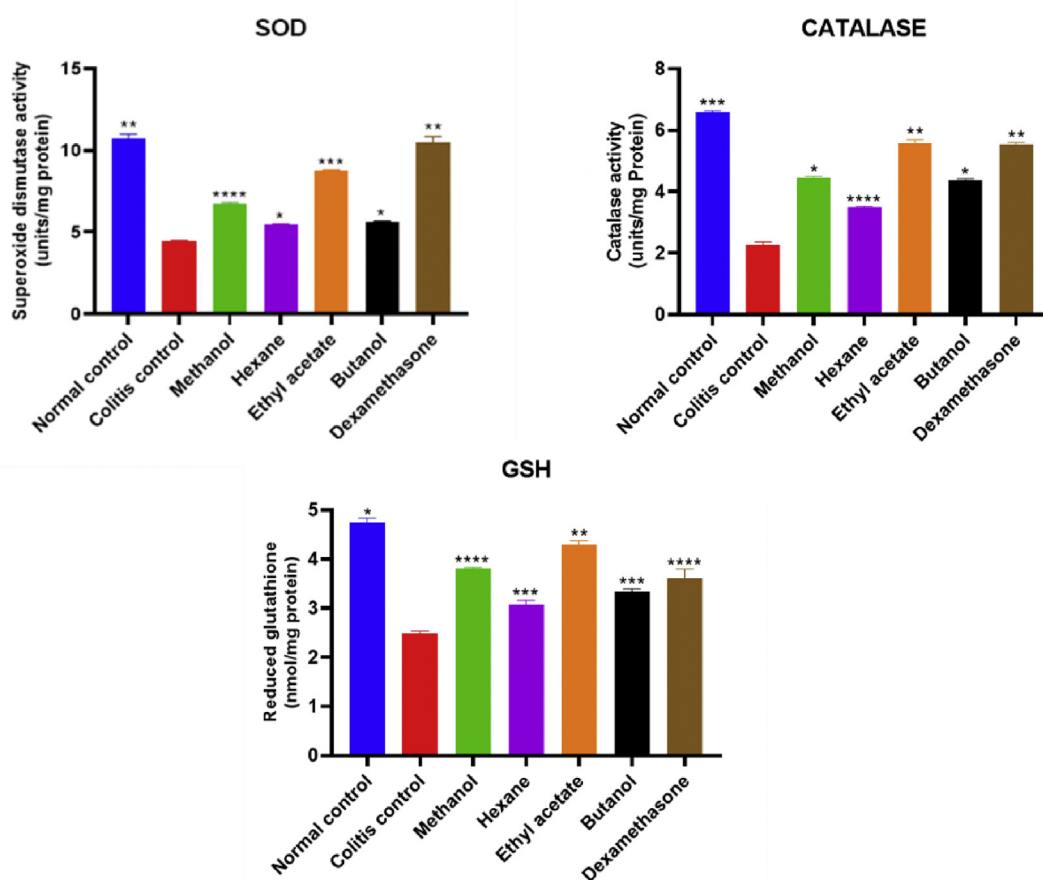
Figure 1. Effect of *P. nivosus* leaf extract and fractions on superoxide dismutase activity (SOD), catalase activity and reduced glutathione (GSH) concentration during acetic acid-induced ulcerative colitis in rats. Values carrying different numbers of asterisks (*) are significantly different ($p > 0.05$).
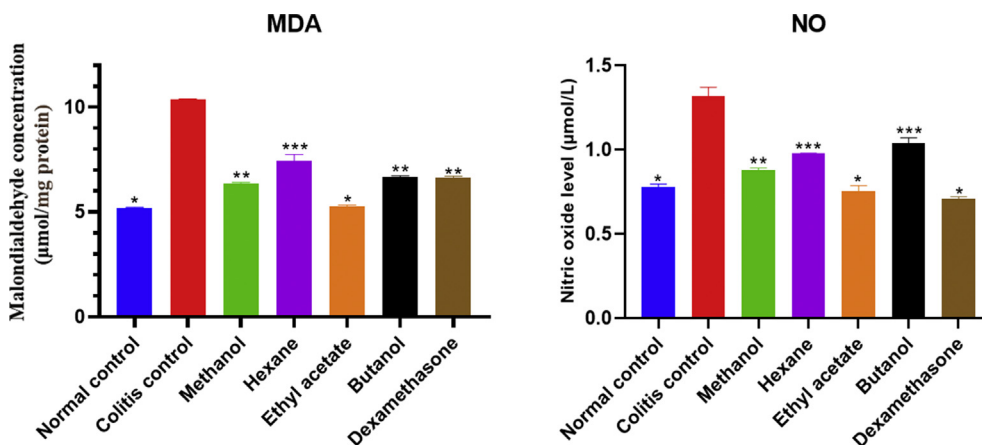
Figure 2. Effect of *P. nivosus* leaf extract and fractions on malondialdehyde and nitric oxide levels during acetic acid-induced ulcerative colitis in rats. Values carrying different numbers of asterisks (*) are significantly different (p > 0.05).
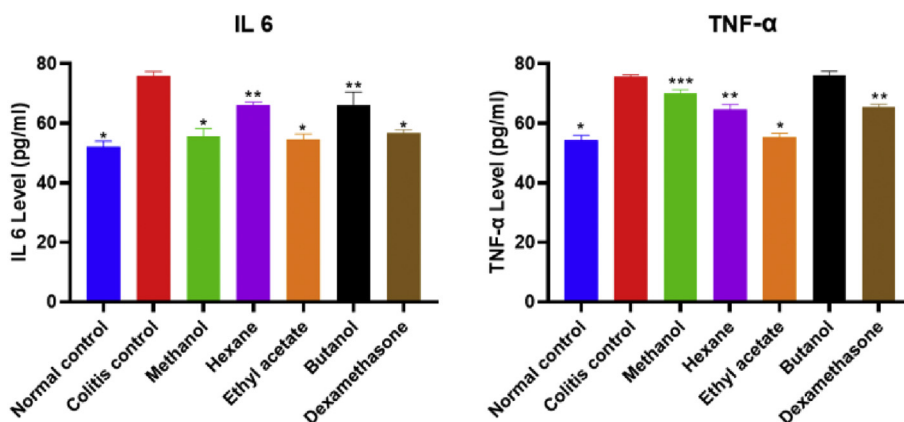

Figure 3. Effect of *P. nivosus* leaf extract and fractions on serum levels of Interleukin – 6 and Tumor necrosis factor-α. Values carrying different numbers of asterisks (*) are significantly different (p > 0.05).

,Z,Z,Z interacted with COX-2 with docking scores ≥ -7.3 Kcal/mol, which is the binding energy of the standard ligand rofecoxib (Table 3). Ethyl iso-allocholate exhibited the highest binding affinity for caspase-1, cholest-22-ene-21-ol, 3,5-dehydro-6- methoxy-, pivalate demonstrated the highest binding affinities for ADRB2 and TNF-α, while alpha-cadinol gave the highest docking score for COX-2. These compounds interacted

with the amino acids at the binding pockets of the respective standard ligands (Figures 5, 6, 7, and 8).

4. Discussion

Intra-rectal administration of acetic acid to rats in this study altered oxidative stress and inflammatory biomarkers as shown by the significant
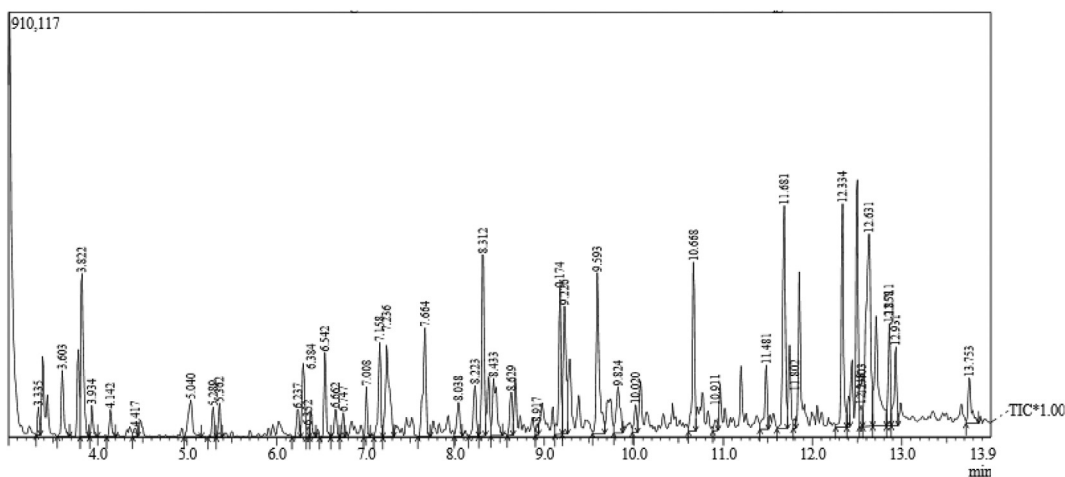

Figure 4. GC-MS chromatogram of ethyl acetate fraction of *P. nivosus* leaf extract.

Table 2. – Chemical composition of the ethyl acetate fraction of *P. nivosus* leaf extract.

Peak	Retention time	Percentage (%) Area (A)	Percentage (%) Height (H)	A/H	Name of compound
1	3.335	0.64	0.88	1.61	beta.-Pinene
2	3.603	1.73	2.09	1.82	alpha.-Phellandrene
3	3.822	4.81	5.19	2.04	D-Limonene
4	3.934	0.69	0.99	1.52	3-Pentanol
5	4.142	0.7	0.86	1.78	Oxirane
6	4.417	0.3	0.3	2.19	cis-3-Hexenyllactate
7	5.04	1.83	1.18	3.42	Benzoic acid
8	5.289	0.7	0.96	1.59	1-Dodecanol
9	5.362	0.88	1.09	1.77	Dodecane
10	6.237	0.77	0.88	1.93	Furane-2-carboxylic acid
11	6.352	0.26	0.34	1.67	Tridecane
12	6.384	0.69	0.86	1.78	Naphthalene
13	6.542	2.04	2.7	1.67	2-Oxabicyclo[2.2.2]octan-6-ol
14	6.662	0.82	0.89	2.01	Cyclohexane, 1,5-diisopropyl-2,3-dimethyl
15	6.747	0.65	0.77	1.84	Bicyclo[4.1.0]heptan-3-ol, 4,7,7-trimethyl
16	7.008	1.17	1.6	1.61	Acetate,(2,4a,5,8a-tetramethyl-1,2,3,4,4a,7,8, 8a-octahydro-1-naphthalenyl) ester
17	7.158	2.64	3.01	1.93	1-Pentadecene
18	7.236	4.24	2.92	3.19	Tetradecane
19	7.664	3.86	3.48	2.44	β-caryophyllene
20	8.038	1.19	1.08	2.43	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z
21	8.223	1.59	1.61	2.17	2,10,10-Trimethyltricyclo[7.1.1.0(2,7)] undec-7-en-6-one
22	8.312	5.11	5.76	1.95	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl
23	8.433	2.75	1.83	3.32	3-Buten-2-ol, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl
24	8.629	1.37	1.37	2.21	Isoledene
25	8.917	0.25	0.37	1.52	Ethyl iso-allocholate
26	9.174	3.72	4.65	1.76	1-Nonadecene
27	9.226	4.18	4.08	2.25	1H-Cycloprop[e]azulen-7-ol, decahydro-1,
28	9.593	5.41	5.13	2.32	Humulane-1,6-dien-3-ol
29	9.824	2.05	1.47	3.08	.alpha.-Cadinol
30	10.02	0.82	0.88	2.04	Cholest-22-ene-21-ol, 3,5-dehydro-6- methoxy-, pivalate
31	10.668	4.19	5.39	1.71	1-Nonadecene
32	10.911	0.4	0.35	2.56	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
33	11.481	1.94	2.03	2.1	Pentadecanoic acid, 13-methyl-, methyl eser
34	11.681	6.75	7.09	2.1	n-Hexadecanoic acid
35	11.802	0.26	0.31	1.8	1-Nonadecene
36	12.334	5.68	7.11	1.76	1-Docosanol
37	12.403	0.76	0.98	1.71	9,12,15-Octadecatrienoic acid, methyl ester
38	12.543	0.53	0.67	1.73	Phytol
39	12.631	10.14	6.13	3.64	Dichloroacetic acid, tridec-2-ynyl ester
40	12.711	5.19	3.5	3.27	Octadecanoic acid
41	12.858	2.46	3.25	1.67	9-Hexacosene
42	12.931	2.28	2.51	2	Acetic acid n-octadecyl ester
43	13.753	1.58	1.46	2.38	9-Hexacosene

reduction in the values of SOD, CAT, and GSH and the significant increase in the levels of lipid peroxidation product (MDA), NO and inflammatory biomarkers - TNF-α and IL-6 (Figures 1, 2, 3, 4, and 5). Intra-rectal ingestion of acetic acid has been reported to induce inflammation and ulcers in the rectum and colon of rats which pathologically resembles human ulcerative colitis [27, 28]. Exposure of the colon to acetic acid leads to the production of acetate ions and subsequent intracellular acidification, which causes damage to the epithelial cells with a corresponding rise in inflammatory responses [29]. Under normal condition, superoxide dismutase protects the body from oxidative damage by converting highly reactive superoxide anion (O_2^{-}) to hydrogen peroxide (H_2O_2) which is broken down by catalase and glutathione peroxidase to water and oxygen [30]. However, during intestinal inflammation, excess production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) causes an alteration in the antioxidant defence system (SOD, CAT, and GSH) [31] and an imbalance in the levels of oxidants and antioxidant system leads to oxidative stress, a major factor in the pathogenesis of ulcerative colitis and the consequent colitis-associated colorectal cancer (CAC). Treatment with antioxidants is therefore suggested as an alternative intervention. Natural products are rich sources of antioxidants; hence, the ameliorative activity demonstrated by *P. nivosus* leaf in this study is highly justified. Treatment with the extract and fractions of *P. nivosus* leaf reversed the observed alterations with the ethyl-acetate fraction displaying the highest antioxidant and anti-inflammatory activity.

The significant reduction in lipid peroxidation levels of experimental rats by *P. nivosus* leaf is an indication of reduced oxidative damage to the intestinal mucosa. Lipid peroxidation is characterized by increased production of lipid peroxides like malondialdehyde (MDA) which are major secondary factors of oxidative damage in UC pathogenesis [32]. Hence,

Table 3. Binding affinities of *P. nivosus* leaf compounds for the anti-inflammatory protein targets.

Compounds	PubChem CID	ΔG Energy (Kcal/mol)			
		Caspase - 1	ADRB2	COX-2	TNF-α
Standard ligands*					
Pralnacasan	153270	-9.0			
Alprenolol	204665		-6.2		
Rofecoxib	5090			-7.3	
Thalidomide	5426				-6.6
beta.-Pinene	440967	-4.9	-5.8	-5.1	-4.4
alpha.-Phellandrene	443160	-5.0	-6.3	-6.6	-4.6
D-Limonene	440917	-4.7	-5.7	-6.7	-4.5
3-Pentanol	11428	-3.2	-3.9	-4.1	-3.4
Oxirane	6354	-2.0	-2.2	-2.3	-2.0
cis-3-Hexenyllactate	5364231	-4.3	-5.7	-5.6	-4.2
Benzoic acid	243	-4.4	-6.0	-5.7	-5.7
1-Dodecanol	8193	-3.6	-5.5	-5.3	-3.8
Dodecane	8182	-3.4	-5.3	-4. 9	-3.7
Furane-2-carboxylic acid	6919	4.1	-5.3	-5	-4.0
Tridecane	12388	-3.8	-5.8	-5.5	-3.9
Naphthalene	931	-5.0	-6.8	-6.8	-5.0
2-Oxabicyclo[2.2.2]octan-6-ol	529885	-4.7	-5.6	-5.7	-4.7
Cyclohexane, 1,5-diisopropyl-2,3-dimethyl	566181	-5.6	-5.9	-5.8	-5.5
Bicyclo[4.1.0]heptan-3-ol, 4,7,7-trimethyl	86056	-4.8	-6.2	-6.7	-4.8
Acetate, (2,4a,5,8a-tetramethyl-1,2,3,4,4a,7 8, 8a-octahydro-1-naphthalenyl) ester	600143	-5.7	-6.4	-6.7	-5.7
1-Pentadecene	25913	-4.0	-5.7	-5.6	-3.7
Tetradecane	12389	-3.5	-5.5	-5.6	-3.7
β-caryophyllene	5281515	-5.1	-6.7	-6.6	-5.9
1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z	5368784	-5.9	-6.5	-7.3	-5.6
2,10,10-Trimethyltricyclo[7.1.1.0(2,7)] undec-7-en-6-one	584518	-6.1	-6.2	-6.5	-5.4
1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl	91104	-5.9	-6.8	-6.7	-5.4
3-Buten-2-ol, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl	90720	-5.8	-6.5	-6.1	-5.5
Isoledene	530426	-6.3	-7.0	-7.3	-5.9
Ethyl iso-allocholate	6452096	-6.5	-7.5	-7.7	-6.1
1H-Cycloprop[e]azulen-7-ol, decahydro-1, 1,7-trimethyl-4-methylene	522266	-5.9	-6.8	-7.2	-5.8
Humulane-1,6-dien-3-ol	5353015	-5.7	-6.4	-6.8	-5.8
alpha.-Cadinol	6431302	-6.2	-7.0	-7.9	-6.0
Cholest-22-ene-21-ol, 3,5-dehydro-6- methoxy-, pivalate	5365019	-5.8	-8.2	-5.4	-6.3
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	5366244	-5.2	-5.6	-5.4	-5.4
Pentadecanoic acid, 13-methyl-, methyl ester	554151	-4.2	-5.1	-5.2	-4.0
n-Hexadecanoic acid	985	-4.1	-6.9	-5.1	-4.1
1-Nonadecene	29075	-3.8	-5.0	-6.3	-4.1
1-Docosanol	12620	-4.8	-5.2	-6.4	-4.1
9,12,15-Octadecatrienoic acid, methyl ester	5367462	-5.1	-5.2	-6.1	-4.4
Phytol	5280435	-4.8	-5.5	-6.0	-4.4
Dichloroacetic acid, tridec-2-ynyl ester	531238	-5.0	-5.0	-5.1	-4.0
Octadecanoic acid	5281	-3.9	-5.6	-6.6	-4.3
9-Hexacosene	5363630	-3.6	-5.0	-7.0	-4.1
Acetic acid n-octadecyl ester	69968	-4.1	-5.5	-6.9	-3.9

*   Standard ligands: Pralnacasan for caspase-1, Alprenolol for ADRB2, Rofecoxib for COX-2, Thalidomide for TNF-α

regulation of lipid peroxide (MDA) production, GSH levels and activities of SOD and CAT by *P. nivosus* leaf as observed in this study could offer an effective therapeutic intervention for IBD.

Nitric oxide (NO) and pro-inflammatory cytokines (TNF-α and IL-6) are major factors for the development of IBD and UC. The significant increase in the levels of these parameters in the serum of AA exposed rats in this study correlates with the results obtained from previous reports. Active inflammation of the colon lining was found to be associated with a rise in the production of inflammatory cytokines which induce iNOS, the enzyme responsible for NO production [33, 34]. Increased levels of these

cytokines during UC could lead to inflammation-induced tumorigenesis and colitis-associated colorectal cancer (CAC) [35]. In this study, treatment with *P. nivosus* leaf extract and fractions significantly reduced the serum levels of NO, IL-6 and TNF-α, thereby suggesting the potential ability of the plant to regulate inflammatory responses which could also prevent the progression of UC to CAC.

The anti-inflammatory activity demonstrated by *P. nivosus* leaf was further validated by the detection of some bioactive compounds in its ethyl-acetate fraction through GC-MS and molecular docking analyses. The compounds demonstrated varying levels of binding affinities for the
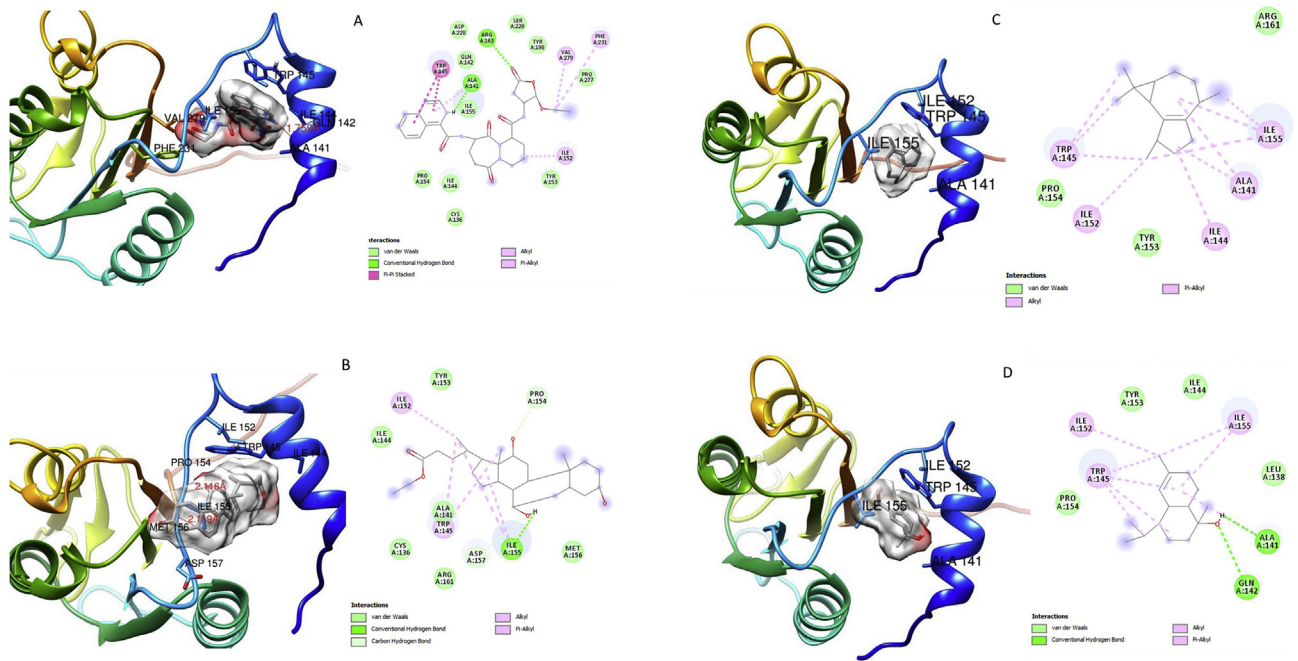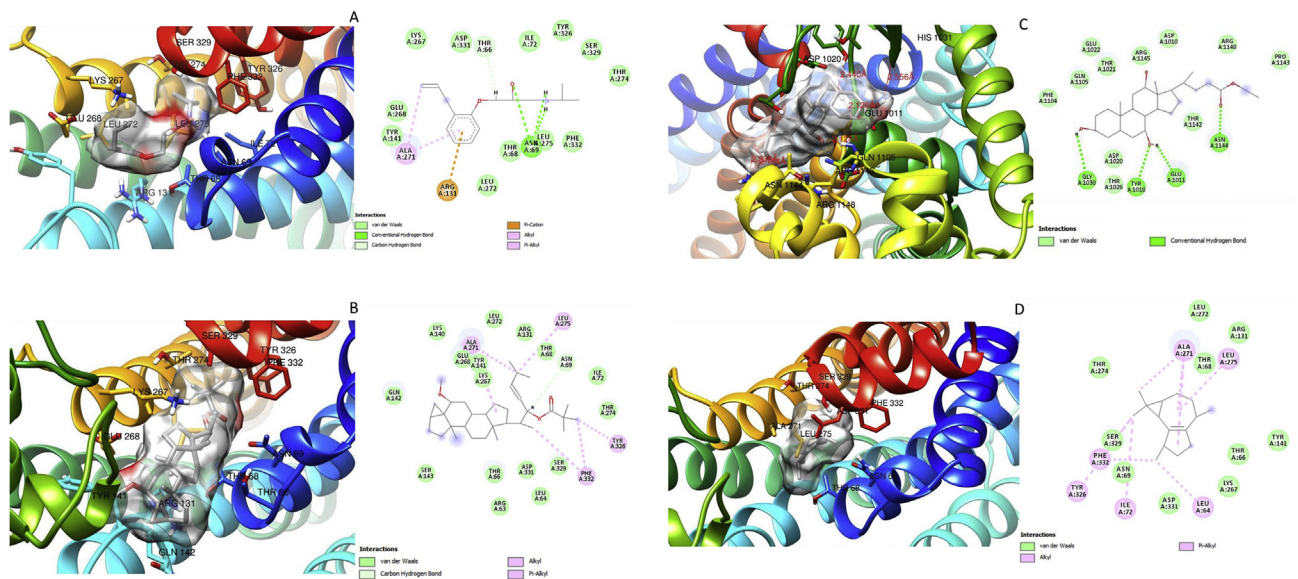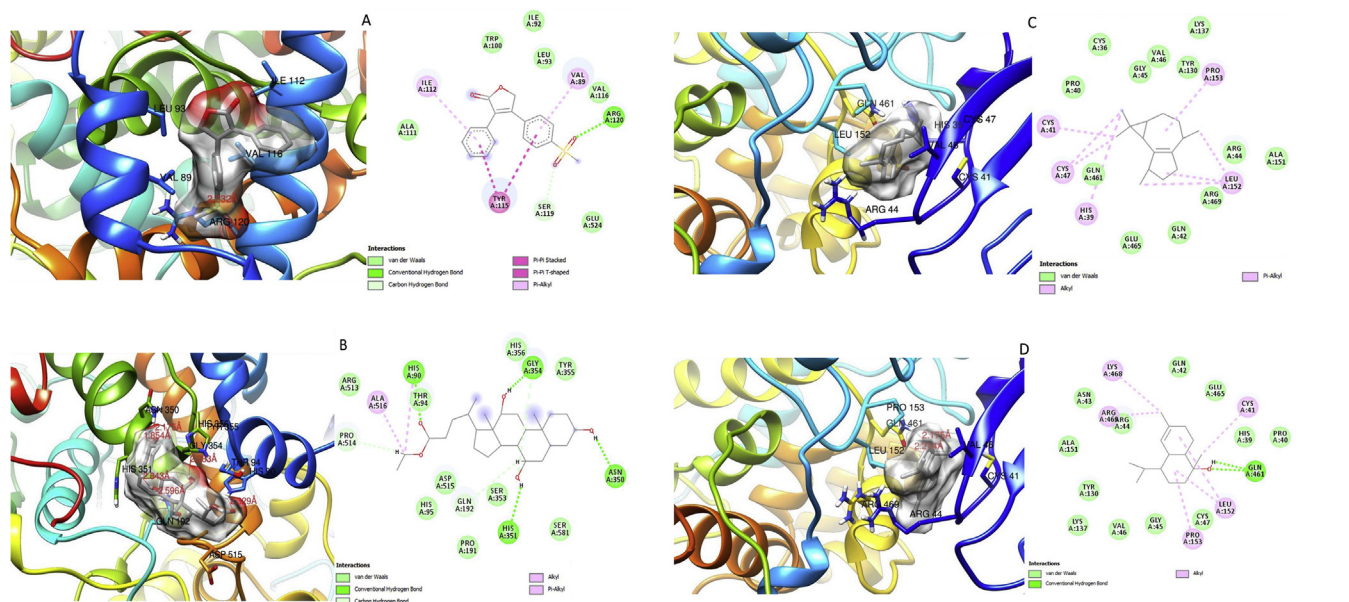
Figure 5. 3D (left) and 2D (right) views of the molecular interactions of amino-acid residues of caspase-1 with (A) Pralnacasan (B) Ethyl iso-allocholate (C) Isoledene (D) alpha-Cadinol.

protein targets, some giving docking scores higher than those of the standard ligands. Molecular interactions with critical proteins of the pro-inflammatory pathways could modulate their activities and subsequently regulate inflammatory responses. Caspase-1 (interleukin (IL)-1-converting enzyme) is responsible for converting pro-interleukin (IL)-1β to its active form (IL-1β). IL-1β is a key pro-inflammatory cytokine secreted in response to tissue injury, diseases or infections and often leads to deleterious consequences. Down-regulation of IL-1β activity through inhibition of caspase-1 is a promising therapeutic intervention against disastrous inflammatory responses [36]. ADRB2 is a G protein-coupled receptor which plays a bidirectional role in immunomodulation and inflammatory responses [37]. Activation of adrenergic receptors could result in pro- and/or anti-inflammatory (regulatory) actions depending

on some factors [38]. Molecular Interaction with this receptor could, therefore, regulate its immunomodulatory activity and subsequently modulate inflammatory responses, which is essential in the management of IBD. COX-2, the molecular target of many non-steroidal anti-inflammatory drugs (NSAIDs) is a member of the cyclooxygenases (COXs) family of enzymes that catalyse the rate-limiting step in the synthesis of inflammatory mediators – prostaglandins [39]. Tumor necrosis factor-alpha (TNF-α) is another regulator of inflammation, and TNF-α inhibitors are reported to be effective in treating inflammatory diseases [40]. Hence, the anti-inflammatory potential of *P. nivosus* leaf could be linked with the ability of its compounds to interact with and possibly regulate the activities of these protein targets.

Figure 6. 3D (left) and 2D (right) views of the molecular interactions of amino-acid residues of ADRB2 with (A) Alprenolol (B) Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate (C) Ethyl iso-allocholate (D) Isoledene.
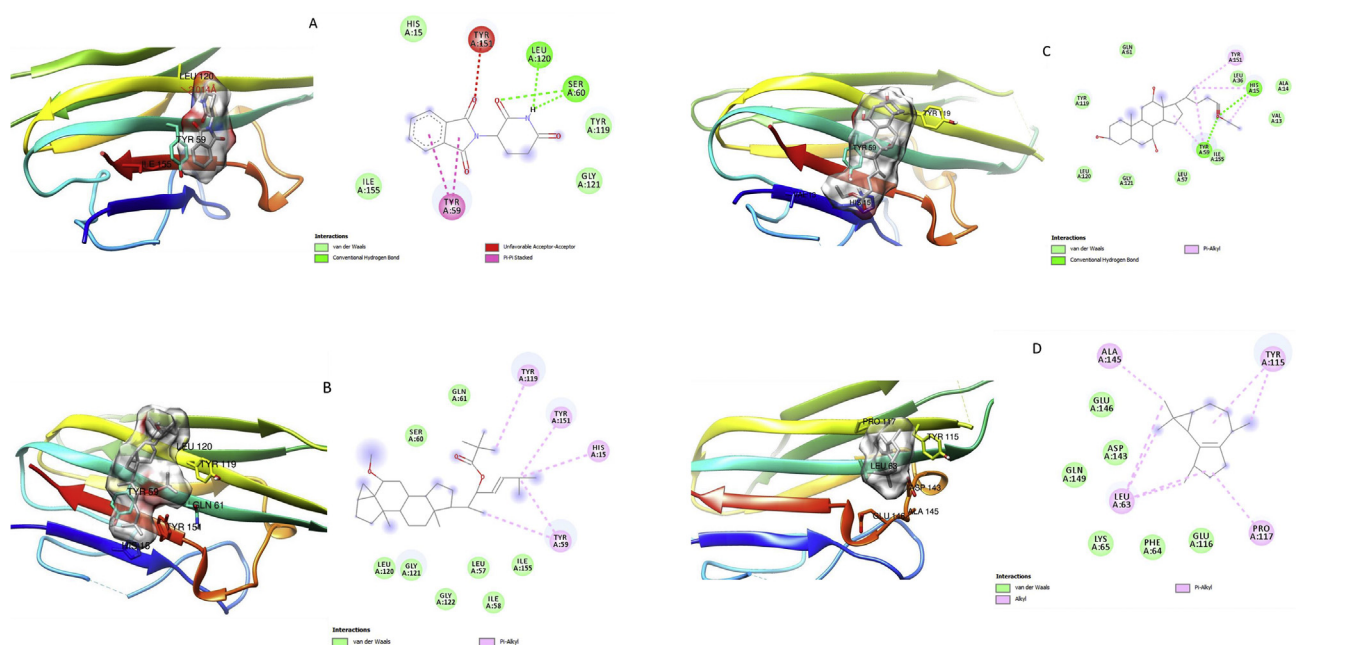
Figure 7. 3D (left) and 2D (right) views of the molecular interactions of amino-acid residues of COX-2 with (A) Rofecoxib (B) Ethyl iso-allocholate (C) Isoledene (D) alpha-Cadinol.

Ethyl iso-allocholate interacted strongly with all the protein targets especially caspase-1. This compound is a steroidal derivative with several therapeutic activities including anti-inflammatory and anti-tumour activities [41, 42]. Its anti-inflammatory activity could be linked with its interaction and possible modulation of these protein targets. Isoledene also interacted strongly with the protein targets, binding to ALA A:141 and other amino acids at the pralnacasan binding site of caspase-1. Isoledene is a terpene reported to induce cytotoxic activities against human colorectal carcinoma cell lines through modulation of caspases and ROS levels [43]. Cholest-22-ene-21-ol, 3,5-dehydro-6- methoxy-, pivalate which occupied the same binding pockets as the standard ligands and exhibited highest binding affinities for ADRB2 and TNF-α is a steroidal derivative with antimicrobial, anti-inflammatory, antiarthritic,

antidiuretic and antiasthmatic activities [44]. Alpha-cadinol with highest binding affinity for COX-2 forms hydrogen bond with GLN A:461 and several van der Waals and alkyl bonds with other amino acids at the binding pocket. This compound is a sesquiterpenoid alcohol reported to demonstrate excellent anti-inflammatory activities in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages [45].

There are other compounds in the extract that exhibited considerable binding affinities for the molecular targets, even though lower than those mentioned above but have been found from literature and PubChem database to possess excellent anti-inflammatory activities. These include phytol, β-caryophyllene and D-limonene. Phytol is a natural linear diterpene alcohol and has been found in previous studies to possess anti-inflammatory and redox-protective activities [46, 47]. This compound

Figure 8. 3D (left) and 2D (right) views of the molecular interactions of amino-acid residues of TNF-α with (A) Thalidomide (B) Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate (C) Ethyl iso-allocholate (D) Isoledene.

was reported to significantly reduce inflammation in mice by inhibiting the recruitment of immune cells; decreasing cytokines and MDA concentrations; and increasing GSH level [46]. This observation agrees with the results obtained in this study. β-caryophyllene is a natural bicyclic sesquiterpene known as a non-steroidal anti-inflammatory agent. Its ability to inhibit cyclooxygenase activity, which is in line with the molecular docking result reported in this study and subsequent inhibition of prostaglandin synthesis and inflammatory responses have been demonstrated [48]. D-Limonene is a natural cyclic monoterpene with reported activity against colitis in rats and a potential anti-inflammatory supplement in humans [49].

5. Conclusions

Phyllanthus nivosus leaf demonstrated anti-inflammatory activity in rats by restoring serum levels of TNF-α, IL-6, NO, MDA, GSH and activities of SOD and CAT. Furthermore, bioactive compounds of the ethyl acetate fraction of *P. nivosus* leaf showed various levels of binding affinities and molecular interactions with anti-inflammatory protein targets - caspase-1, ADRB2, COX-2 and TNF-α - thereby validating the anti-inflammatory potential of the plant.

Declarations

Author contribution statement

T.O. Johnson, K.D. Odoh, A.O. Akinanmi and A.E. Adegboyega: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

C.O. Nwonuma: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

[1] J. Cosnes, C. Gower-Rousseau, P. Seksik, A. Cortot, Epidemiology and natural history of inflammatory bowel diseases, Gastroenterology 140 (2011) 1785–1794.

[2] R. Jegadeesan, X. Liu, M.R. Pagadala, N. Gutierrez, M. Butt, U. Navaneethan, Microscopic colitis: is it a spectrum of inflammatory bowel disease? World J. Gastroenterol. 19 (26) (2013) 4252–4256.

[3] A.A. Awaad, H.F. Alkanhal, R.M. El-Meligy, G.M. Zain, V.D. Sesh Adri, D.A. Hassan, et al., Anti-ulcerative colitis activity of Calotropis procera Linn, Saudi Pharmaceut. J. 26 (1) (2018) 75–78.

[4] S. Hyams, R.E. Behrman, P.M. Kliegman, A.M. Arvin, Inflammatory bowel diseases, in: Nelson Textbook of Paediatrics, WB Saunders Co, Philadelphia, 2004, pp. 1575–1585.

[5] I.O. Senbanjo, K.A. Oshikoya, C.A. Onyekwere, F.B. Abdulkareem, O.F. Njokanma, Ulcerative colitis in a Nigerian girl: a case report, BMC Res. Notes 5 (2012) 564.

[6] C.N. Bernstein, F. Shanahan, Disorders of a modern lifestyle: reconciling the epidemiology of inflammatory bowel diseases, Gut 57 (9) (2008) 1185–1191.

[7] K. Goh, S.D. Xiao, Inflammatory bowel disease: a survey of the epidemiology in Asia, J. Dig. Dis. 10 (1) (2009) 1–6.

[8] H. Akiho, A. Yokoyama, S. Abe, Y. Nakazono, M. Murakami, Y. Otsuka, K. Fukawa, M. Esaki, Y. Niina, H. Ogino, Promising biological therapies for ulcerative colitis: a review of the literature, World J. Gastrointest. Pathophysiol. 6 (4) (2015) 219–227.

[9] K.L. Wallace, L.B. Zheng, Y. Kanazawa, D.Q. Shih, Immunopathology of inflammatory bowel disease, World J. Gastroenterol. 20 (2014).

[10] G. Rogler, Chronic ulcerative colitis and colorectal cancer, Cancer Lett. 345 (2) (2014) 235–241.

[11] F. Ke, P.K. Yadav, L.Z. Ju, Herbal medicine in the treatment of ulcerative colitis, Saudi J. Gastroenterol. 18 (1) (2012) 3–10.

[12] F.S. Karawya, E.A. Metwally, Effects of jujube fruit extract on acetic acid-induced colitis in adult male rats, J. Exp. Clin. Anat. 15 (2016) 19–26.

[13] World checklist of selected plant families. https://wcsp.science.kew.org/furtherInformation.do?name_id=24399. (Accessed 10 June 2019).

[14] T.O. Johnson, I. Gurumtet, P.U. Ugwu, K.M. Nkom, D.C. Okafor, D. Oleru, et al., In vitro Biochemical evaluation of the antiplasmodial activities of various fractions obtained from Phyllanthus nivosus leaf extract, in: Proceedings of ISERD International Conference, World Research Library, Putrajaya, Malaysia, 2017, pp. 1–4.

[15] O.R. Ezeigbo, I. Nwachukwu, C.A. Ike-Amadi, J.B. Suleiman, Evaluation of the phytochemical, proximate and mineral constituents of *Breynia nivosa* leaf, Int. J. Biochem. Res. Rev. 20 (2) (2017) 1–8.

[16] E.S. Amadi, C.A. Oyeka, R.A. Onyeagba, O.C. Ugbogu, I. Okoli, Antimicrobial screening of Breynia nivosus and ageratum conyzoides against dental caries organisms, J. Biol. Sci. 7 (2) (2007) 354–358.

[17] F.A. Onyegbule, I.O. Ilouno, C. Ikeh, B.O. Umeokoli, P.M. Eze, Evaluation of phytochemical constituents, analgesic, anti-inflammatory, antimicrobial and antioxidant activities of extracts of Breynia nivosa leaves, Planta Med. (2014) LP18–LP80.

[18] T.O. Johnson, I. Gurumtet, R.J. Kutshik, In vitro and in vivo analysis of the antiplasmodial activity of ethanol extract of Phyllanthus nivosus W. Bull leaf, J. Parasit. Dis. 44 (4) (2020) 166–173.

[19] W.N. Arifin, W.M. Zahiruddin, Sample size calculation in animal studies using resource equation Approach, Malays. J. Med. Sci. 24 (5) (2017) 101–105.

[20] A.D. Millar, D.S. Rampton, C.L. Chander, A.W. Claxson, S. Blades, A. Coumbe, J. Panetta, C.J. Morris, D.R. Blake, Evaluating the antioxidant potential of new treatments for inflammatory bowel disease using a rat model of colitis, Gut 39 (3) (1996) 407–415.

[21] M. Madesh, K.A. Balasubramanian, Microtitre plate assay for superoxide dismutase using MTT reduction by superoxide, Indian J. Biochem. Biophys. 35 (1998) 184–188.

[22] A.K. Sinha, Colorimetric assay of catalase, Anal. Biochem. 47 (2) (1972) 389–394.

[23] M.S. Moron, J.W. Depierre, B. Mannervik, Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver, Biochim. Biophys. Acta 582 (1) (1979) 67–78.

[24] K.A. Balasubramanian, M. Manohar, V.I. Mathan, An unidentified inhibitor of lipid peroxidation in intestinal mucosa, Biochim. Biophys. Acta 962 (1) (1988 Sep) 51–58.

[25] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids, Anal. Biochem. 126 (1) (1982 Oct) 131–138.

[26] O. Trott, A.J. Olson, Autodock Vina: improving the speed and accuracy of docking with new scoring function, efficient optimization, and multithreading, J. Comput. Chem. (2) (2010) 455–461.

[27] Y.F. Cagin, H. Parlakpinar, N. Vardi, A. Polat, Y. Atayan, M.A. Erdogan, et al., Effects of dexpanthenol on acetic acid-induced colitis in rats, Exp. Therap. Med. 12 (5) (2016) 2958–2964.

[28] R. Fabia, R. Willén, A. Ar'Rajab, R. Andersson, B. Ahrén, S. Bengmark, Acetic acid-induced colitis in the rat: a reproducible experimental model for acute ulcerative colitis, Eur. Surg. Res. 24 (4) (1992) 211–225.

[29] D. Amit, P. Anagha, G. Anamika, M. Anwesha, S. Sandipan, S. Antara, et al., Ameliorative effect of ferulic acid against acetic acid induced ulcerative colitis: role of HO-1 and Nrf2, Pharmacologia 7 (2016) 114–124.

[30] T. Ikumoto, S. Hayashi, S. Tomita, S. Miwa, H. Mitomi, T. Fujimori, et al., Manganese superoxide dismutase plays an important role in the inflammatory process and predicts disease severity and activity in patients with ulcerative colitis, APMIS Acta Pathol. Microbiol. Immunol. Scand. 122 (6) (2014) 512–517.

[31] S.V. Rana, S. Sharma, K.K. Prasad, S. Sinha, K. Singh, Role of oxidative stress & antioxidant defence in ulcerative colitis patients from north India, Indian J. Med. Res. 139 (2014) 557–568.

[32] Z. Wang, S. Li, Y. Cao, X. Tian, R. Zeng, D.F. Liao, D. Cao, Oxidative stress and carbonyl lesions in ulcerative colitis and associated colorectal cancer, Oxid. Med. Cell. Long. (2016) 9875298.

[33] W.E. Roediger, M.J. Lawson, S.H. Nance, B.C. Radcliffe, Detectable colonic nitrite levels in inflammatory bowel disease mucosal or bacterial malfunction? Digestion 35 (1986) 199–204.

[34] N. Avdagić, A. Zaćiragić, N. Babić, M. Hukić, M. Seremet, O. Lepara, E. Nakaš-Ićindić, Nitric oxide as a potential biomarker in inflammatory bowel disease, Bosn. J. Basic Med. Sci. 13 (1) (2013) 5–9.

[35] S. Grivennikov, E. Karin, J. Terzic, D. Mucida, G.Y. Yu, S. Vallabhapurapu, et al., IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer, Cancer Cell 15 (2) (2009) 103–113.

[36] A. Denes, G. Lopez-Castejon, D. Brough, Caspase-1: is IL-1 just the tip of the ICEberg? Cell Death Dis. 3 (2012).

[37] L. Wu, Y. Tai, S. Hu, M. Zhang, R. Wang, W. Zhou, J. Tao, Y. Han, Q. Wang, W. Wei, Bidirectional role of β2-adrenergic receptor in autoimmune diseases, Front. Pharmacol. 9 (2018) 1313.

[38] M. Sharma, P.M. Flood, Adrenergic Receptors as Pharmacological Targets for Neuroinflammation and Neurodegeneration in Parkinson's Disease, Neuroprotection, Raymond Chuen-Chung Chang and Yuen-Shan Ho, IntechOpen, 2018.

[39] M. Diederich, C. Sobolewski, C. Cerella, M. Dicato, L. Ghibelli, The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies, Int. J. Cell Biol. 2010 (2010).

[40] E. Esposito, S. Cuzzocrea, TNF-alpha as a therapeutic target in inflammatory diseases, ischemia-reperfusion injury and trauma, Curr. Med. Chem. 16 (2009) 3152–3167.

[41] P. Singariya, P. Kumar, K.K. Mourya, Isolation of some new steroids and evaluation of bio-activity of Cenchrus ciliaris, Int. J. Res. Pharm. Sci. 3 (2012) 678–684.

[42] R.S. Thakur, B. Ahirwar, ScienceDirect A steroidal derivative from Trigonella foenum graecum L . that induces apoptosis in vitro and in vivo, J. Food Drug Anal. 27 (2018) 231–239.

[43] M. Asif, A. Shafaei, S.F. Jafari, S.K. Mohamed, M.O. Ezzat, A.S. Abdul Majid, et al., Isoledene from Mesua ferrea oleo-gum resin induces apoptosis in HCT 116 cells through ROS-mediated modulation of multiple proteins in the apoptotic pathways: a mechanistic study, Toxicol. Lett. 257 (2016) 84–96.

[44] S. Arora, G. Kumar, S. Meena, Gas chromatography-mass spectroscopy analysis of root of an economically important plant, Cenchrus ciliaris L . from Thar desert, Rajasthan (India) 10 (2017) 64–69.

[45] Y. Tung, P. Yen, C. Lin, S. Chang, Y. Tung, P. Yen, et al., Anti-inflammatory activities of essential oils and their constituents from different provenances of indigenous cinnamon (Cinnamomum osmophloeum) leaves, Pharm. Biol. 48 (2010) 1130–1136.

[46] R.O. Silva, F.B. Sousa, S.R. Damasceno, N.S. Carvalho, V.G. Silva, F.R. Oliveira, D.P. Sousa, K.S. Aragão, A.L. Barbosa, R.M. Freitas, J.V. Medeiros, Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress, Fundam. Clin. Pharmacol. 28 (4) (2014) 455–464.

[47] N.D. Phatangare, K.K. Deshmukh, V.D. Murade, Isolation and characterization of phytol from Justicia gendarussa Burm. f.-An anti-inflammatory compound, Int. J. Pharm. Phytochem. Res. 9 (6) (2017) 864–872.

[48] National Center for Biotechnology Information, PubChem database. Beta-B-caryophyllene, CID=5281515, https://pubchem.ncbi.nlm.nih.gov/compound/5281515. (Accessed 29 April 2019).

[49] P.A. d'Alessio, R. Ostan, J. Bisson, J.D. Schulzke, M.V. Ursini, M.C. Béné, Oral administration of d-Limonene controls inflammation in rat colitis and displays anti-inflammatory properties as diet supplementation in humans, Life Sci. 92 (24–26) (2013) 1151–1156.