Bioactive and Functional Groups Revealed by GC-MS and IR in *Acmella Caulirhiza* Modulate Inflammation and Nucleation (*In Vitro* Models): Implication in Kidney Stone Management

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ABSTRACT

Inflammation is linked to many diseases including urolithiasis or kidney stone formation. Anti-inflammatory drugs are increasing in demand due to the rising number of inflammatory linked diseases like kidney stone. Therefore, this study was undertaken to evaluate the *in vitro* anti-inflammatory and anti-urolithiasis ability of *Acmella caulirhiza* extract. The results showed a dose dependent inhibition fashion in all assays presented. Therefore, at 100 µg/ml concentration of *A. caulirhiza* or aspirin, the percentages inhibition of heat induced hemolysis were 97.48 ± 0.98 % and 98.91 ± 1.01 % respectively. *A. caulirhiza* inhibited hypotonic solution induced hemolysis and albumin denaturation. The extract inhibited trypsin at 9.65 ± 0.28 and diclofenac at 20.08 ± 1.38 % at 200 µg/ml concentration. The extract also inhibited the enzyme lipoxygenase activity and the production of thiobarbituric acid reactive substances (TBARS). The extract also inhibited nucleation *in vitro* at 70.32 ± 1.13 for *A. caulirhiza* and 87.33 ± 1.71 % for cystone at concentration of 1000 µg/ml. The phytochemicals, IR and GC-MS analysis revealed the presence of phenolics, flavonoids and alkaloids. The IR also revealed functional groups present in phenolics, flavonoids and alkaloids and other secondary metabolites and the presence of phytol, hexadecanoic acid, squalene and other phytoconstituents showed that the extract is a potential candidate for an anti – inflammatory and anti-kidney stone formation in vitro.

Keywords: *Acmella caulirhiza*, Inflammation, Kidney Stone, Phytochemicals, GC-MS.

INTRODUCTION

Inflammation is a protection mechanism to an injury caused by microbes and other stressors. Part of this inflammatory process involves denaturation of protein, increased blood flow to the

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inflamed site and membrane disintegration [1]. At the end of this protection mechanism the body eliminate the stressor and return the body back to homeostasis. Inflammation entails a series of cascade marked by four cardinal signs of heat, redness, pain and swelling. The mechanism of prostaglandin synthase is central to the inflammatory process. Prostaglandin and thromboxane acts as inflammatory regulators [2]. These chemicals can lead to increased flow of blood at injury site, and recruitment of many inflammatory cells. Uncontrolled and excessive inflammation can lead to irreparable damage even though inflammation is a protective mechanism it can be more dangerous than the injury [2]. Non- steroidal anti-inflammatory drugs used against inflammatory processes possess adverse effects, irritation of gastric lining and ulcers. Hence there is the need for the search of safe, cheap, readily available and clinically effective drugs. That is why the research was sought to investigate the efficacy of Acmella cualirhiza extract in vitro. This was done using different models of anti-inflammatory processes, such as protein denaturation; heat induced hemolysis, Hypotonic induced hemolysis, trypsin inhibition, inhibition of lipoxygenase and lipid peroxidation inhibition.

Kidney stone is made of calcium oxalate (CaOx) hard materials that form in one or both of the kidneys due to the presence of certain minerals in the urine [3]. Calcium that is not metabolized by the bones and muscles goes to the kidneys and is passed out as urine, but when this process fails, kidney stones are precipitated [4]. The process of kidney stones formation is complex and is influenced by many factors such as urine concentration, pH, phosphate, certain calcium [5]. The process of kidney stones formation may begin with saturation, nucleation, crystal growth, aggregation and obstruction [6]. Obstruction can give rise to severe pain, frequent urination and blood in the urine [7]. Treatment and management of kidney stones are based on the ability of the drugs to reduce or eliminate the size of the stone. Thiazide diuretics and alkaline citrate are used frequently for treatment and management of kidney stones [8]. Other treatments include endoscopic stone removal and ureteroscopy. These procedures are very sophisticated, not common, unsafe and expensive and many cannot afford them [9]. On the other hand, medicinal plants like *Acmella caulirhiza* abundant in Nigeria with phytomedical properties can be an alternative.

A. caulirhiza is a flowering plant found in subtropical African countries. It is mostly found in Nigeria especially in humid soil and wasted areas. It belongs to the family Asteraceae. *A. caulirhiza* is called the "toothache plant" due to its tingling and numbing sensation, if flowers and buds are chewed. It has analgesic potential [10]. Most of the properties exhibited by *A. caulirhiza* were attributed to a chemical compound known as spilanthol an alkaloid [11]. *A. caulirhiza* is used for the treatment of many common ailments afflicting developing African countries such as toothache, ulcers of the mouth and sore throats [12]. Studies have also showed antibacterial activity of A. cualirhiza extract against certain pathogenic bacteria and fungi [13]. Infusions made from *A. caulirhiza* are used in central Kenya for the alleviation of ear, nose and throat aches [14]. The fresh leaves juice of A. cualirhiza and water is a medicine for stomach pain and diarrhea [15-16].

MATERIALS AND METHOD

A. caulirhiza preparation

The flowers of *A. caulirhiza* were plucked, washed and shade dried for two weeks. Later they were extracted with absolute methanol, with intermittent stiring for 72 hrs. The extract was concentrated, stored at 4^0C in an air tight amber colored bottle for further use.

Phytochemical screening

Qualitative phytochemical determination was carried out on the extract of *A. caulirhiza* to determine the presence of alkaloids, flavonoids, tannins, phenols and saponins according to the methods of [17-18].

Gas chromatography and mass spectrometry analysis

The extracted sample of *A. Caulirhiza* 1µl was injected into the equipment at a column temperature of 300C and heated to 3000C. The carrier gas was Helium, voltage for ionization of compound was 70eV. The mass spectrometry scan was between 45-450MHz. The unknown compounds were searched in Spectrometer data base of NIST [19].

Infrared spectroscopy analysis

The dried flower of *A. Caulirhiza* extract was mixed with potassium bromide and was subjected to infrared at 500 to 4000cm-1 interval.

Isolation of erythrocytes

Healthy human volunteer's blood was drawn and centrifugrd for 10 min at 3000 rpm. The supernantant was discarded and the erythrocytes were washed thrice with equal volume

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of normal saline. Erythrocytes were suspended in 10% (v/v) normal saline for further use.

Heat induced hemolysis

In a 2 ml reaction mixture containing *A. caulirhiza* extract or Aspirin as reference (100 – 1000 µg/ml) and 1 ml of 10 % (v/v) erythrocytes in centrifuge tubes. The tubes were later warmed at 56^{0}C for 30 min. at the end of the mild heating, tubes were centrifuged at 3000 rpm for 10 min and optical density was taken at 560 nm [20].

Hypotonicity induced hemolysis

The mixture consisted of RBC (0.5 ml), 5 ml (10 mM NaCl in 10 mM phosphate buffered saline) and *A. caulirhiza* or the reference drug at different concentrations. The control was devoid of extract or reference. All reaction tubes were kept at room temperature for 10 min, and later centrifuged at 3000 rpm and absorbance of supernatant was read at 540 nm. Antihemolysis was calculated as percentage [21].

Albumin denaturation inhibition assay

This method was adopted from [22]. A mixture of 0.45 ml of 5 % albumin (human source), *A. caulirhiza* extract or diclofenac. The control tubes were devoid of extract or reference drug. The pH of the mixture was adjusted to 6.3 with 1 M HCl and later incubated at 37^{0}C for 20 min and thereafter at 57^{0}C for 3 min. Phosphate buffered saline was then added (2.5 ml) and turbidity was measured at 416 nm.

Trypsin inhibitory assay

The procedure of [23] was applied in this study. A reaction of 2000 µl contained trypsin 0.6 µg, 1 ml of *A. caulirhiza* extract at 100 – 1000 µg/ml and 1000 µl of tris-HCl buffer 25 mM at a pH of 7.4. The reaction was placed in a water bath at 37 0C for 5 min and casein 0.8 % w/v was added as substrate. The mixture was kept in the water bath for 20 min. later 2000 µl of of perchloric acid 70 % v/v was added to halt the enzyme activity. The mixture was centrifuged at 500 rpm for 5 min, absorbance of supernatant was read at 280 nm. Diclofenac served as standard and results were processed as percentages.

Anti-lipoxygenase activity

A. caulirhiza inhibitory activity against lipoxygenase was analyzed based on the method of [24]. *A. caulirhiza* and indomethacin as standard were dissolved in 2 M borate buffer solution pH 9.0 at different concentrations and 0.25 ml of lipoxygenase enzyme (2000 U/ml) were incubated for 5 min at 25^{0}C. later, substrate was included, agitated and absorbance was measured at 234 nm. Percentage inhibittion was calculated for *A. caulirhiza* and indomethacin.

Thiobarbituric acid (TBA) assay

Lipid peroxidation assay contained erythrocytes 20 % incubated with 20 mM H_2O_2 for 60 min at 37^{0}C in the presence of A. caulirhiza. Thereafter tubes were added 1.5 ml of 10 % TCA and centrifuged for 10 min. the supernatant was mixed with 1.5 ml of 0.67 % TBA in 50 % acetic acid. The reaction mixture was then heated for 30 min at 90 ^{0}C [25]. The developed pink color was read at 535 nm. Lipid peroxidation was expressed as percentage. Quercetin served as standard.

***In Vitro* Anti-kidney stones formation using nucleation assay**

Anti-kidney stones formation ability of *A. caulirhiza* extract was analysed. Extract at different concentration was directed towards calcium oxalate (CaOx) crystallization utilizing spectrophotometry. Tris buffer 0.05M constituting $CaCl_2$, CaOx and NaCl was warmed at 37 ^{0}C for 30 min. Thereafter the optical density of the tubes was measured at 620 nm [26]. The percentage anti-nucleation was then calculated.

Statistical analysis

All the data were processed and presented as mean ± S.D, using excel and GraphPad prism software.

RESULTS

Phytochemical result

Saponins	++
Tannins	+
Phenolics	+++
Flavonoids	++
Alkaloids	+
Phlobatanins	-

key: + present; - Absent

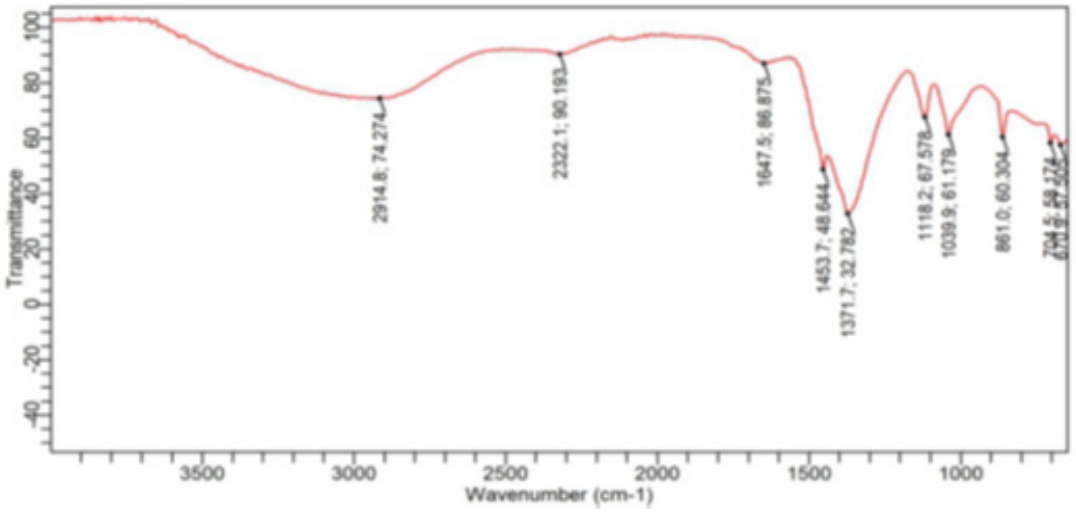

Figure 1. Showing Fourier transform infrared spectroscopy of *A. caulirhiza* flower extract.

The IR spectra revealed that the sample had an absorption at 2914.8 cm-1 is due C-H stretch, at 1647 is due to carbonyl group or free amide, 1453.7 cm-1, is C=C unsaturated group. While 1371 cm-1 is due to CH2 bending. These reflect that most of the chemical constituents are n-alkyl, and unsaturated derivative of organic compounds.

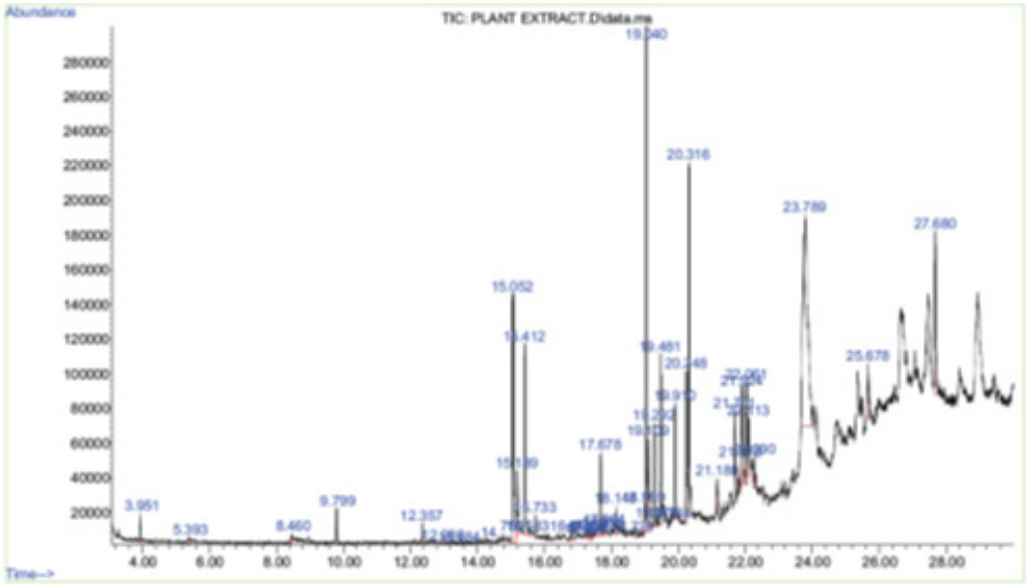

Figure 2. Showing the GC-MS chromatogram of *A. caulirhiza* flower extract.

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Table 3. Compounds detected in GC-MS analysis of methanolic extracts of A. caulirhiza

Peak	Name of compound	Retention time	Area%
1	2,3-dimethyl-heptane	3.951	0.61
2	1, 1-[(1-methyl-1, 3-propanediyl)bis(oxymethylene)]bis-Benzeneethanol, .alpha., beta. –dim ethyl-	5.393	0.19
3	Cis-2,6-Nonadien-1-ol 3-Hexyn-1-ol	8.460	0.30
4	2,2-Dimethyl-1-(2,4,6-trimethylphenyl)propane-1-one	9.799	0.70
5	3-methyl-5-propyl	12.357	0.34
6	5-methyl-Nonyl tetradecyl ether	12.986	0.18
7	1-ethenyl-1,1,3,3-tetramethyl-3-(2-propenyl)- 4-Hydroxy-4-methyl-4H-naphthalen-1	13.484	0.17
8	1-bromo-Tetradecane	14.760	0.19
9	8-epoxybenzo[7]annulene Dihydrocoumarin	15.052	7.56
10	2-methyloctacosane	15.189	1.82
11	2,4-Di-tert-butylphenol Phenol	15.412	3.94
12	Tricosylpentafluoropropionate	15.533	0.20
13	Octacosane	15.733	0.53
14	2,6,10,14-tetramethyl-2-Bromotetradecane	16.843	0.38
15	3,8-dimethyl-Sulfurous acid	17.278	0.17
16	2,6,10-trimethyl-	17.329	0.22
17	octadecyl vinyl ester	17.421	0.37
18	Silane	17.570	0.46
19	Heneicosane	17.678	2.01
20	Dotriacontane	17.770	0.23
21	Butyl dotriacontyl ether	17.804	0.32
22	2-Bromo dodecane	18.148	0.58
23	2,6,10-trimethyl-Oxalic acid	18.725	0.19
24	3,7,11,15-tetramethyl-2-Hexadecene	18.989	0.62
25	2,6,6-trimethyl-Bicyclo[3.1.1]heptane	19.040	12.32
26	3,7,11,15-tetramethyl-2-Hexadecene	19.109	2.77
27	3,7,11,15-Tetramethyl-2-hexadecen- 1-ol 1,4-Eicosadiene	19.292	2.34
28	5-ethyl-1,3-dioxan-5-yl isobutyl ester	19.378	0.25
29	1-ethynylcyclohexanol	19.481	3.94
30	2,4,6-trimethyl Decane	19.784	0.23
31	Tetracosane	19.910	2.74
32	n-Hexadecanoic acid	20.248	4.10
33	Dibutyl phthalate	20.316	7.28
34	(1S,2R,4R,7R)-4-Isopropyl-7-methyl-3,8-dioxatricyclo[5.1.0.02,4]octane	21.180	0.77
35	Phytol	21.701	2.19
36	Methoxyacetic acid	21.873	0.50
37	Hentriacontane	21.924	2.86
38	Dimethyl(dimethylpentyloxysilyloxy)hexyloxy-Silane	22.061	2.45
39	Octadecanoic acid	22.113	1.34
40	Tetrapentacontane	22.290	0.85
41	1,1'-(2-propyl-1,3-propanediyl)bis-cyclohexane	23.789	23.82
42	Benzo[h]quinoline-2,4-dimethyl	25.678	1.82
43	Squalene	27.680	5.17

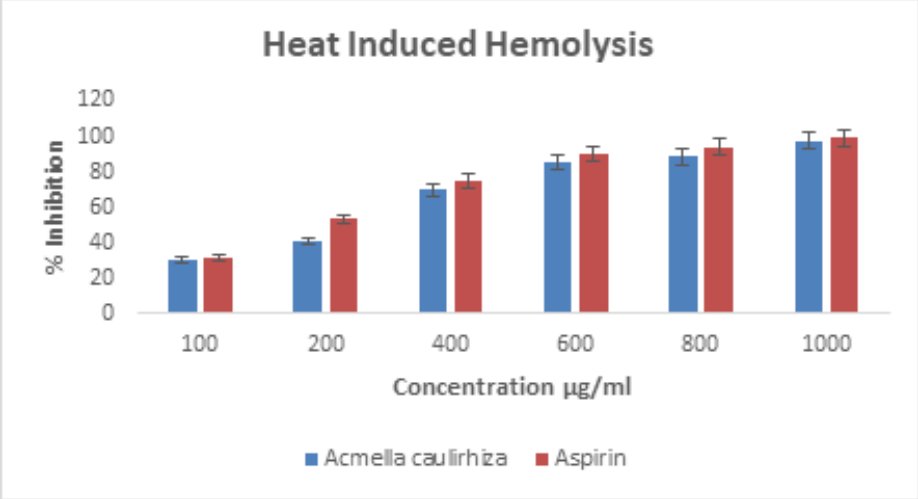

Figure 3. Showing heat induced hemolysis of erthrocytes, in the presence of *A. caulirhiza* and the standard drug (aspirin) at different concentrations. The pattern of inhibition was concentration dependent. At dose of 100 µg/ml *A. caulirhiza* inhibited 29.93 % while the standard drug inhibited 31.43 %. There was significant difference between the plant extract and the standard drug (P<0.05).

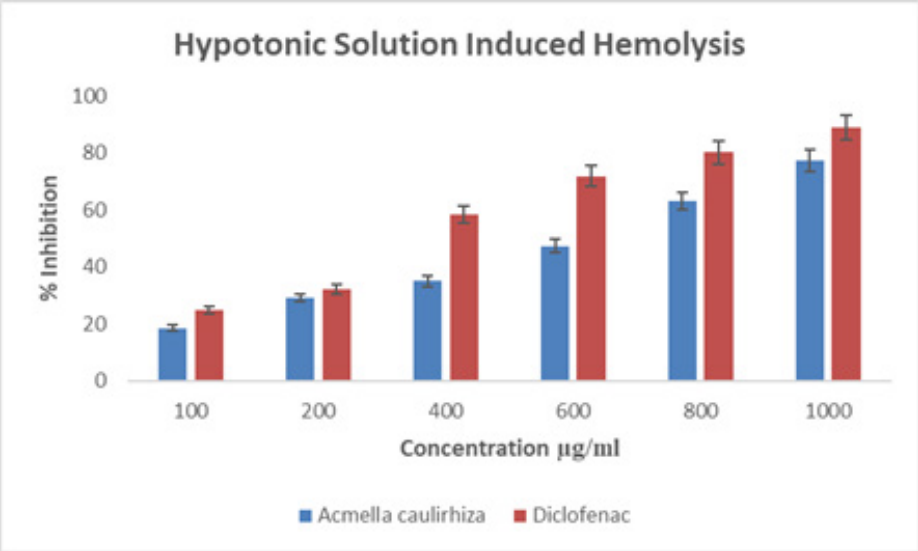

Figure 4. Showing hypotonic solution induced hemolysis of erthrocytes, in the presence of *A. caulirhiza* or the standard drug (diclofenac) at different. The pattern of inhibition was concentration dependent. At a dose of 100 µg/ml *A. caulirhiza* inhibited 18.61 % while the standard drug inhibited 25.01 %. There was significant difference between the plant extract and the standard drug (P<0.05).

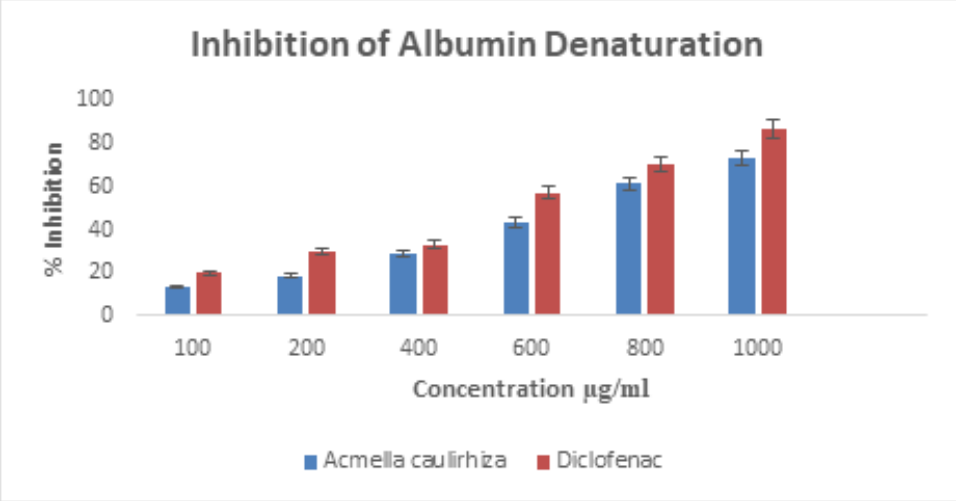

Figure 5. Showing inhibition of albumin denaturation, in the presence of *A. caulirhiza* and the standard drug (diclofenac) at different concentrations. The pattern of inhibition was concentration dependent. At a dose of 100 µg/ml *A. caulirhiza* inhibited 13.21 % while the standard drug inhibited 20.16 %. There was significant difference between the plant extract and the standard drug (P<0.05).

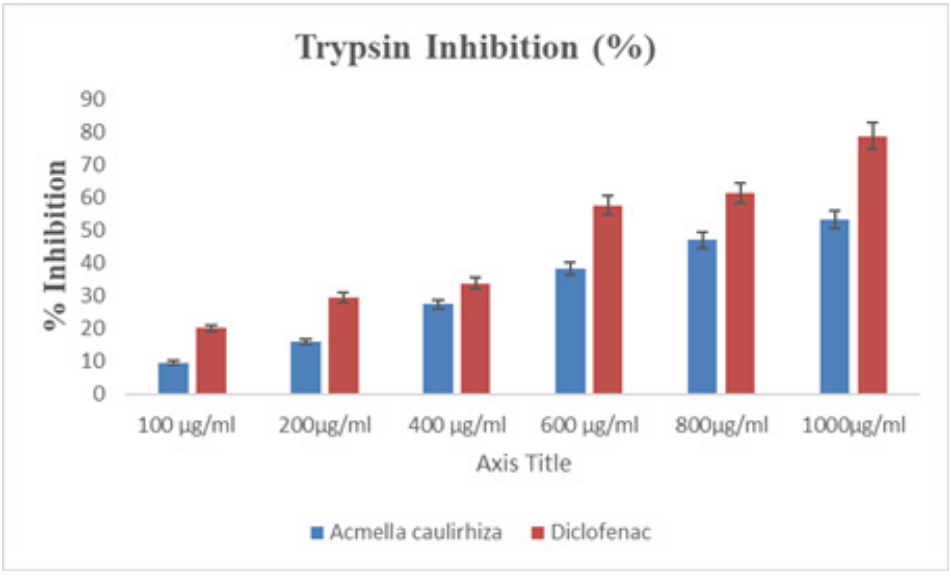

Figure 6. Showing % inhibition of trypsin, in the presence of *A. caulirhiza* and the standard drug (diclofenac) at different concentrations. The pattern of inhibition was concentration dependent. At a dose of 100µg/ml *A. caulirhiza* inhibited 9.65 % while the standard drug inhibited 20.08 %. There was significant difference between the plant extract and the standard drug (P<0.05).

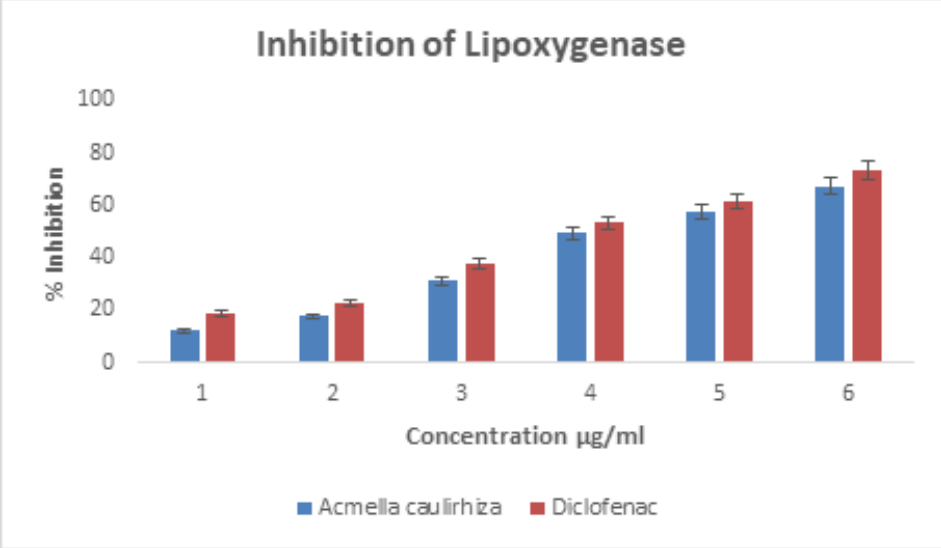

Figure 7. Showing % inhibition of lipoxygenase, in the presence of *A. caulirhiza* and the standard drug (diclofenac) at different concentrations. The pattern of inhibition was concentration dependent. At a dose of 100µg/ml *A. caulirhiza* inhibited 12.11 % while the standard drug inhibited 18.63 %. There was significant difference between the plant extract and the standard drug (P<0.05).

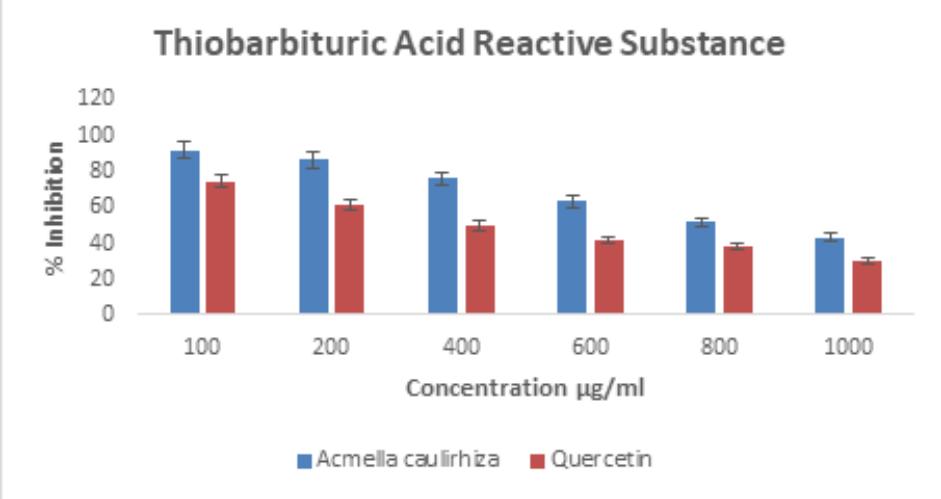

Figure 8. Showing % inhibition of thiobarbituric acid reactive substance, in the presence of *A. caulirhiza* or the standard drug (quercetin) at different concentrations. The pattern of inhibition was concentration dependent. At a dose of 100µg/ml *A. caulirhiza* inhibited 91.87 % while the standard drug inhibited 74.71 %. There was significant difference between the plant extract and the standard drug (P<0.05).

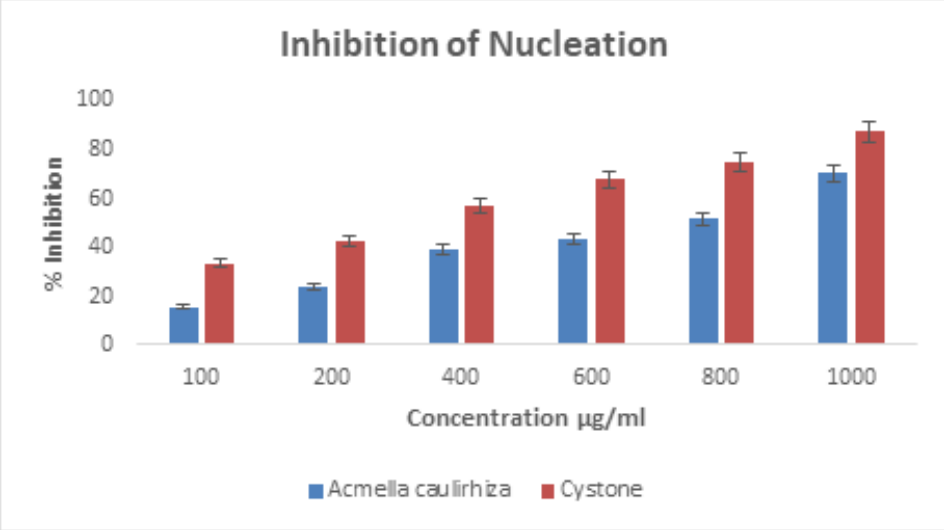

Figure 9. Showing % inhibition of nucleation, in the presence of *A. caulirhiza* and the standard drug (cystone) at different concentrations. The pattern of inhibition was concentration dependent. At a dose of 100µg/ml *A. caulirhiza* inhibited 15.63 % while the standard drug inhibited 33.22 %. There was significant difference between the plant extract and the standard drug ($P<0.05$).

DISCUSSION

Qualitative phytochemical determination was carried out to detect the presence of secondary metabolites. In the present study, *A. caulirhiza* extract revealed the presence of phenolics, flavonoids and alkaloids. The IR revealed functional groups like C=C, C-H, CO. CNH and CH2 these groups revealed the presence of phenols, flavonoids and alkaloids that are found in the extract of A. caulirhiza. These results are similar to the findings of [27], who also reported functional groups in the extract of Capparis divaricate. The GC-MS analysis of extract revealed a lot of abundant biocompounds which are 43 in number but those that are more abundant are squalene, benzo[h]quinolone-2,4-dimethyl, octadecanoic acid, phytol, hexadecanoic acid, 2,4-ditertbutyl phenol, 8-epoxybenzo [7] annulene dihydrocoumarin, dibutyl phthalate etc. these compounds have been reported to have antioxidant, anticancer, anti-inflamatory, antibacterial and antidiabetic properties [28-31].

Protein denaturation involves disruption of forces that stabilizes secondary, tertiary and quaternary levels of protein arrangement which could lead to the death of cells. Denaturing agents include heat, salt, acidity, stress etc. denaturation of proteins lead to inflammation and in turn to cancer, ulcer, cataract etc. [32]. To investigate the mechanism underlying the ability of *A. caulirhiza* to act as anti-inflammatory drug, the extract was subjected to protein denaturation inhibition assay in vitro. The result showed that extract has potentials to inhibit denaturation as showed in figure 5 above; this ability of the extract could be due to the phytochemicals present in the extract of *A. caulirhiza* that can regulate the activities of auto-antigens [33]. These results are also like the reports of [34] who also reported anti-albumin denaturation by the extract of Murraya koenigii.

Protection of the membrane of lysosomes is key to anti-inflammatory processes, because lysis of the lysosomal membrane which resembles erythrocyte membrane leads to inflammation [35]. Hypotonicity and heat induced hemolysis may be by squeezing the cell membrane due to osmotic discharge of cellular electrolyte and fluid [36]. The ability of extracts to inhibit this process in erythrocytes is by either preventing damage to the membrane or efflux of intracellular components [37]. The components in these extracts are responsible for this inhibitory activity, which are phytochemicals. Many active compounds were found in *A. caulirhiza* which are also responsible for the maintenance of erythrocyte membrane integrity when induced by heat or hypotonic solution. Our reports are similar to the reports of [35].

Protease inhibitors are useful drugs for the treatment of many inflammatory related diseases like rheumatoid arthritis, cancer, AIDS, thrombosis and pancreatitis [38]. Proteases are rich in lysosomes of neutrophils. They play cardinal role in the development of tissue damage during the inflammatory process [39]. In inflammatory processes trypsin is also

activated in the initial process [40], in the present study trypsin is inhibited by *A. caulirhiza* in a concentration dependent fashion. Our reports are similar to the findings of [35,33].

Nucleation is the rate limiting step in the process of kidney stone formation, nucleation begins when there is a combination of stone salt in the urine with other new stones like calcium chloride and calcium oxalate, this then result in calcium oxalate formation [41]. Saponin abundant fraction of the plant extract Herniaria hirsuta was reported to inhibit nucleation *in vitro* [42]. The present phytochemical result of *A. caulirhiza* showed the presence of saponins and the anti-nucleation result revealed that *A. caulirhiza* inhibited calcium oxalate nucleation in vitro. These reports are similar to the work of [43].

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