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RESEARCH ARTICLE

Phytochemical Analysis and Biological Assay of the Methanolic Leaf Extract of CadabafarinosaForsk (Capparidaceae)

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Abstract

Cadabafarinosafarsk (Capparidaceae) is a plant commonly found in arid zones all over the globe and it is used **in** traditional medicine for the treatment of skin and breast cancer in North-Eastern Nigeria. Ethnobotanical survey asserts that root of the plant has been used in cancer treatment. Methanolic leaf extract showed moderate but inconsistent antioxidant property on2, 2- diphenyl- 1- picrylhydrazyl (DPPH) free radical. The same extract however, showed significant cytotoxicity on Brine shrimp larvae, nauplii as it gave percentage mortality above 50% for most of the concentrations which ranged from 10 µg/ml to 1000 µg/ml. Plants with cytotoxic and antioxidant properties have been found to contain anticancer or antitumour compounds

INTRODUCTION

Oral interview has revealed that the people of Bade inNorth-Eastern Nigeria rely much on traditional medication to manage cases of various forms of cancer. One of the main plants in anticancer preparation is *Cadabafarinosa*Forsk (Capparidaceae). One of the interview respondents claimed that there is no type of cancer that *C. farinosa* cannot treat and he was ready to bet the authors in case they doubted his submission. It was on this premise that this present research was based. Orwa et al, (2009) in their documentary, 'Agroforestry database' gave the local names of *Cadabafarinosa*Forssk in Arabic, Fula and Hausa as Suraya, Baggahi and Bagayi respectively. *C. farinosa* belongs to the family Capparidaceae and is widely distributed all over the globe especially in arid area. It was used in folklore medicine as purgative, anthelmintic, antisyphilitic, emmenagogue, aperients, stimulant and many other physiological and pharmacological uses including potency against liver damage, cancer and antioxidant in the body, (Umesh*et al*, 2013). An ethnomedicinal survey of plants used in the folkloric treatment of breast cancer and other related diseases was conducted in three locations of North-Eastern Nigeria, specifically in Maiduguri, Jimeta and Nguru(Mohammed et al, 2014) and *Cadabafarinosa* was implicated only with analgesic and anti-inflammatory effects, but Moshi *et al*, (2006) reported the traditional use of whole root of *C. farinosa* in cancer treatment. In the same report it was asserted that out of 60 plants tested for Brine shrimp cytotoxicity 6 plants including *C. farinosa* were of least activities compared to that of standard drug cyclophosphamide. Although the traditional claim and the experimental findings of Moshi et al, (2006) do not seem to agree, Al-Musayeib*et al*, (2013) isolated a new triterpene ester together with 8 known compounds from its leaves andresult of antioxidant activity proved that the compounds exhibited strong activity compared to a standard drug propyl gallate, a known synthetic antioxidant used for the test. These research reports showed that *C. farinosa* extract not cytotoxic but pure isolate has antioxidant capacity.

MATERIALS AND METHOD

SAMPLE COLLECTION, IDENTIFICATION AND PREPARATION

Identifiable parts of *Cadabafarinosa*Forssk (Capparidaceae) was collected in May, 2014 fromGashua, Yobe state in North Eastern Nigeria and conveyed to the North East Arid Zone Development Programme (NEAZDP), Gashua, where identification was duly made by a plant taxonomist.

The leaves of the plant were collected and shade dried for fourteen days to ensure complete dryness. The dried materials were pulverized in a clean mortar and pestle into coarse powder and stored in a polythene bag which was again enclosed in a polystyrene bag for safety till time of use.

EXTRACTION OF PLANT MATERIAL

The dried plant leaves previously pulverized was subjected to48 hours maceration methanol. This method is used widely in the extraction of plant metabolites because of its convenience. The main advantage is that it is safer for heat labile chemical components but the disadvantage is that it offers low yield and large amount of solvent is consumed in the process and it takes time to recover the solvent.

PHYTOCHEMICAL SCREENING

Phytochemical analysis was performed on the crude extracts using the standard methods of Sofowora, (2008), Evans (2009) and Tiwari*et al*, (2011) for the qualitative screening of phytocmpounds of interest. The phytocmpounds that were screened for are: Alkaloids, Flavonoids, Saponins, tannins, Cardiac glycosides, Anthraquinones, steroids, terpenoids, reducing sugar and phlobatannins.

Test for reducing sugars: Fehling'stest

To about 2ml aqueous solution of the extract in a test tube was added 5ml mixture of equal volumes of Fehling's solution 1 and II and boiled in a water bath for about 2 minutes. The test tube was observed for brick-red precipitate.

Test for Tannins:

Ferric chloride test: To about 2ml of the aqueous solution of the extracts was added few drops of 5% Ferric chloride solution (light yellow). The occurrence of blue-black colour shows the presence of gallic tannins and a green-black colour indicates catechol tannins.

Braymer's test: Few drops of extract was treated with 10% alcoholic $FeCl_3$ solution and observed for bluish green colouration for tannins.

Test for saponins

Frothing test: 3ml of the aqueous extract was mixed with 10ml distilled water in a test tube. The tube was stoppered and shaken vigorously for 5 minutes; it was allowed to stand for 30 minutes and observed for honeycomb froth.

Emulsion test: 3ml of the aqueous extract was dissolved in 10ml distilled water, stoppered and shaken vigorously for 5 minutes, after which 2-3 drops of olive oil were added and shaken again. Then it was put to rest for 30 minutes and observed for a layer of emulsion in between the aqueous layer and froth.

Test for cardiac glycosides

Salkowski's Test. The extract was dissolved in 2ml of chloroform. Concentrated sulphuric acid was carefully added to form a lower layer. A reddish-brown colour at interface indicated steroidal ring (that is aglycone portion).

Leibermann's Test. The extract was dissolved in 2ml of acetic anhydride and cooled well in ice. Concentrated sulphuric acid was added carefully. A colour change from violet to blue to green indicated presence of steroidal nucleus (that is aglycone portion of the cardiac glycoside)

Test for terpenoids/steroids

Leibermann/Burchard's Test. To 2ml of chloroform extract, 2ml of acetic anhydride and few drops of concentrated sulphuric acid were added in a test-tube. Blue-green ring between layers indicated steroids. Pink-purple ring indicatedterpenoids.

Test for flavonoids

Lead Acetate Test

Extracts were treated with few drops of lead acetate solution. Formation of yellow coloured precipitate indicated the presence of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution formation of intense yellow colour which becamecolourless on addition of dilute hydrochloric acid indicated the presence of flavonoids.

Test for Alkaloids

1.0g of extract was dissolved in 5ml of 10% ammonia solution and extracted with 15ml of chloroform. The chloroform portion was evaporated to dryness and the resultant residue dissolved in 15ml of dilute sulphuric acid.

Meyer's reagent test (Bertrand's reagent): 2.3 drops of Meyer's reagent was added to a portion of the acidic solution in a test tube and observed for an opalescence or yellow-white precipitate indicative of the presence of alkaloids.

Dragendorff's reagent: 2ml of acidic solution in the second test tube was neutralized with 10% ammonia solution. Dragendorff's reagent was added and turbidity or precipitate was observed indicative of presence of alkaloids.
Wagner's Test: Few drops of Wagner's reagent were added to the acidic solution of the extract. A reddish- brown precipitate indicated the presence of alkaloids

Test for Phlobatannins (Condensed Tannins)

Aqueous extract was boiled with 1% aqueous Hydrochloric acid. Red precipitate was indicative of phlobatannins

BIOLOGICAL ASSAY

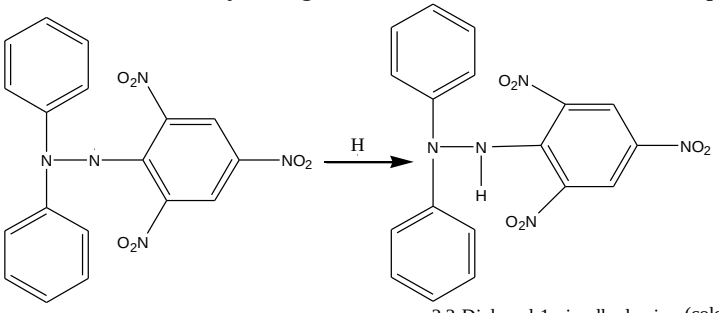
The general screening method was used as was suggested in Vlietinck and Apers (2001) that; a broad screening bioassay is probably the most useful if one is randomly screening chosen organism for any kind of pharmacological activity. In this research the random or general bioassay was employed wherebytwo bioassays were tried on the extracts, as follows:

1. **Antioxidant Assay**

Antioxidants are defined as compounds that delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Oxidative stress is an imbalanced state where excessive quantities of reactive oxygen and/or nitrogen species (ROS/RNS), for example superoxide anion, hydrogen peroxide, hydroxyl radical, peroxynitrite, overcome endogenous antioxidant capacity, leading to oxidation of a variety of biomacromolecules such as enzymes, proteins, DNA and lipids. Oxidative stress is important in the development of chronic degenerative diseases including coronary heart disease, cancer, aging and diabetes mellitus. (Dai and Mumper, 2010)

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Stable Free Radical Scavenging Assay

The presence of antioxidant phytocompounds in the various extracts was determined by their ability to scavenge the 2, 2-Diphenyl-1- picrylhydrazyl (DPPH) stable free radical. DPPH gives a deep purple coloured solution when dissolved in 95% methanol but on reacting with a reducing agent, a hydrogen donor; it becomes colourless or yellowish which is the evidence for the formation of reduced neutral form of DPPH. It is a discoloration assay which is evaluated by the addition of an antioxidant material to a DPPH solution and the ability to scavenge the stable free radical is measured by taking the absorbance of the mixture on a spectrophotometer.



2,2-Diphenyl-1-picrylhydrazyl (purple) 2,2-Diphenyl-1-picrylhydrazine (colourless)

The capacity of the extract fractions to scavenge the stable free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), was monitored according to Soni and Sosa (2013), Marinova and Batchvarov (2011). 2ml of 1mg/ml solutions of each extracts in 95% methanol were mixed with 2ml of 0.1mM methanolic solution of DPPH free radical. The test solution of 1.0 mg/ml was serially diluted to 500 µg/ml, 250 µg/ml, 125 µg/ml and 62.5 µg/ml. The mixtures were vortexedthoroughly for one minute at room temperature and incubated for 30 minutes in the dark. Finally, absorbance of each of the mixtures was read at 517nm on a JENWAY 6305 spectrophotometer. The same experiment was conducted with a negative control or blank constituted of 95% methanol mixed with the DPPH solution and also with a positive control constituted of 1mg/ml of L-(+)-Ascorbic acid dissolved in 95% methanol. The antioxidant capacity of each extract sample and the controls were expressed in terms of percentage inhibition and was calculated as:

$$\%\text{Inhibition} = \frac{Ab - Ac}{Ac} \times 100,$$

Where, Ab = the absorbance of blank,
And Ac = absorbance of extract or control.

2. Cytotoxicity

In a general primary screening for bioactivity,the popular bioassays used include the brine shrimplethality assay (BSLA) and the crown-gall tumour inhibition test (Vlietnick and Apers 2001). The first technique is a bench-top screening method and, was adopted in this research.It is an *invivo* lethalilty test usinga tinycrustacean, which is the brine shrimp (*artemia salina*). Since the introduction of this technique in 1982, this test has been used for the isolation of *in vivo* antitumour agents and pesticides from plant (Vlietinck and Apers, 2001).

In this technique, eggs of the brine shrimp were added to a hatching chamber containing artificial sea water, the chamber was kept under inflorescent lamp for 48hrs for the eggs to hatch into shrimp larvae, that is *nauplii*. 20mg of the methanolic extract of *Cadabafarinose* was dissolved in 2ml of methanol, to serve as stock solution1. From the stock solution, more dilute solutions were prepared by serial dilution. Each concentration was made in triplicate. 0.5ml of each solution was allowed to evaporate completely in sample vials. To each of the evaporated sample vial 4.5ml of artificial sea water was introduced by use of pipette followed by a drop of DMSO to aidsolubilization of test samples in the vials. Approximately ten free-swimming *nauplii*were introduced into each vial by use of Pasteur pipette and the total volume of the sea water was adjusted to 5ml by dropping in more of the sea water from a dropper. Each test sample has 4 serial concentrations of 1000µg/ml, 100µg/ml, 10µg/ml and 1µg/ml. A control (blank) solution was made by taking 5ml of the artificial sea water in a sample vial including a drop of DMSO. Into each sample vial was introduced approximately 10 free-swimming *nauplii* and all of these solutions were allowed to stand for 24 hours after which the number of free-swimming nauplii were counted andthe LC_{50} orpercentage (%) mortality were computatedto give the final cytotoxicities of the test extracts (Adoum*et al*, 2009,Olowa and Nuneza, 2013 and Tawaha, 2006).

LC_{50} is the lethal concentration at which 50% of test organisms were killed and this can also be determined by calculating the percentage mortality from which the lethal concentration can also be determined. LC_{50} of less than 100µg/ml was considered as potent (active), but, according to Olowa and Nuneza (2013), LC_{50} less than 1000µg/ml was toxic while IC_{50} values greater than 1000µg/ml was non-toxic. The percentage mortality (%M) was also calculated by dividing the dead nauplii by the total number and then multiplied by 100. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

RESULTS

Phytochemical Analysis

Phytochemical analysis of the methanolic leaf extrat of *Cadabafarinosa* was performed according to standard methods as stated previously and the outcome was presented in Table 1 below

Table 1: Phytochemicals of *Cadabafarinosa*

Phytochemicals in Cadabafarinosa										
Saponins	Terpenoids	Steroids	Flavonoids	Glycosides Cardiac	Tannins	Phloba-tannins	Anthraqui-nones	Reducing Sugar	Alkaloids
+	+	-	+	-	-	-	-	+	-

Brine Shrimp Lethality Assay (BSLA)

The method of Tawaha (2006) was adopted in this in vivo assay. The Brine Shrimp eggs were hatched according to the procedure and serial dilutions of the extract was prepared to give four different concentrations in triplicate. After free swimming nauplii were introduced into the test solutions it was left for 24 hours after which surviving free swimming nauplii were counted and percentage mortalities calculated and presented on Table 2 below

Table 2: Brine Shrimp Lethality Assay of *Cadabafarinosa*

Concentration of *Cadabafarinosa* (µg/ml) In Triplicate	Number of Nauplii used	Number of Survived Nauplii	Total Survivors	% Mortality
1000	9	1	4	84
1000	7	3		
1000	9	0		
100	8	3	8	64.3
100	10	3		
100	8	2		
10	8	4	10	52.4
10	7	2		
10	6	4		
1	5	4	11	35.3
1	5	3		
1	7	4		
0	6	6	21	12.5
0	10	8		
0	8	7		

Antioxidant Assay

The scavenging capacity of the methanolic leaf extract of Cadabafarinosa on 2, 2-diphenyl-1-picrylhydrazyl free radical was monitored according toMarinova and Batchvarov (2011) and Soni and Sosa (2013) in which test solutions and standard antioxidant, ascorbic acid were prepared to give 1.0 mg/ml and subsequently diluted, while that of DPPH was 0.1 mM solutions all in 95% methanol. Results are presented in Tables 3 and 4 below

Table 3: **Antioxidant assay of Ascorbic acid**

Ascorbic acid Conc In µg/ml	Absorbance (517nm)	% Inhibition
1000	0.141	85.76
500	0.304	69.27
250	0.449	54.64
125	0.502	49.29
62.5	0.556	43.84
Blank	0.990	0.00

Figure 1: Antioxidant Assay of Ascorbic acid (Control drug)

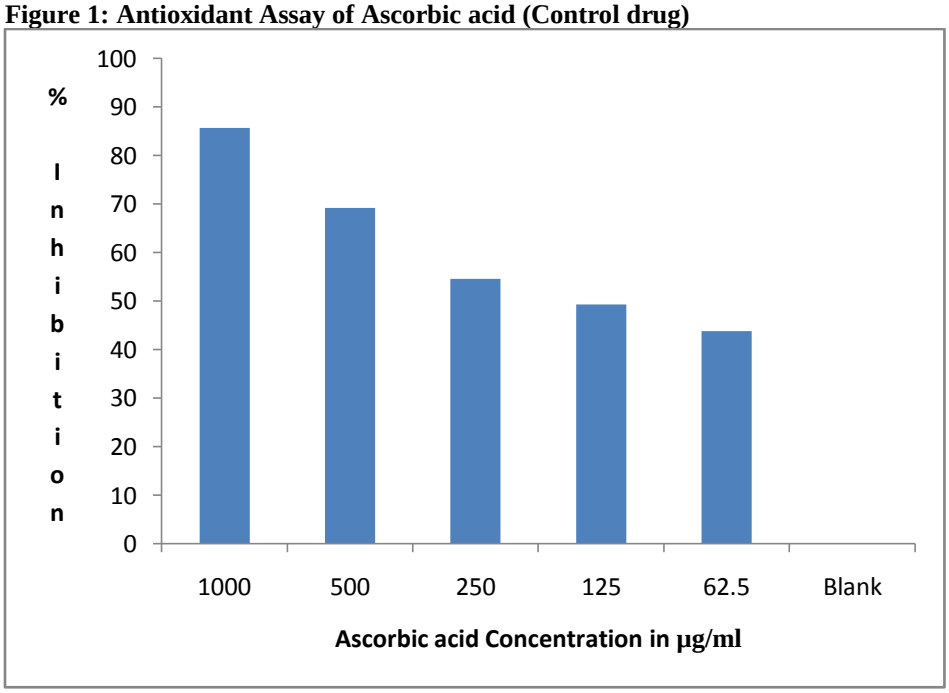
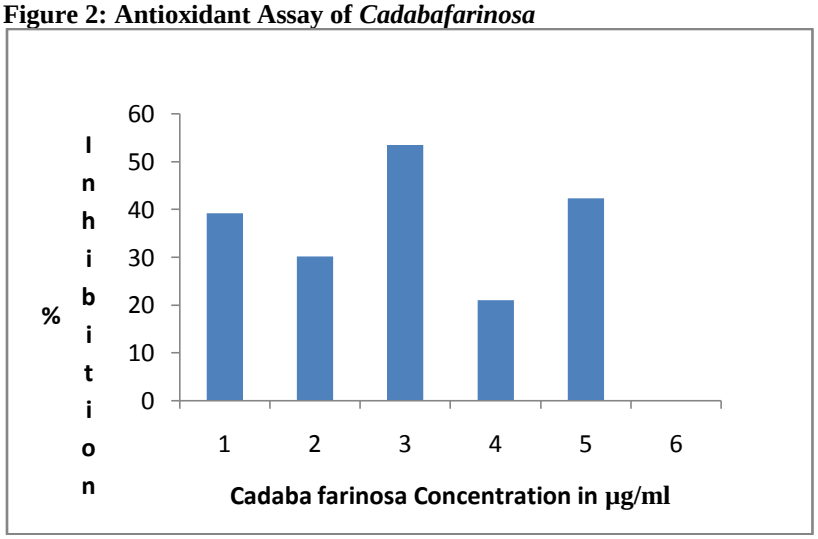


Table 4: Antioxidant Assay of Cadabafarinosa

Cadabafarinosa Extract Concentration In µg/ml	Absorbance (517nm)	% Inhibition
1000	0.602	39.19
500	0.692	30.10
250	0.461	53.43
125	0.782	21.01
62.5	0.572	42.22
Blank	0.990	00.00

Figure 2: Antioxidant Assay of *Cadabafarinosa*



DISCUSSION

Phytochemical screening of the crude methanolic leaf extracts of the *Cadabafarinosa* in Table 1 showed varieties of bioactive secondary metabolites. Whereas the plant extract did not show presence of alkaloids, steroids, phlobatannins and anthraquinones, it showed the presence of flavonoids, terpenoids, saponins and reducing sugar. The presence of flavonoids in the plant is in agreement with previous researches mentioned earlier in the literatures and it is a pointer that this plant could be a good source of antioxidants, anticarcinogens and cytotoxic bioactive compounds. Evans, (2009) and Haraguchi*et al*, (2001) reported flavonoids as protective agents against oxidative damage. In addition Haraguchi*et al*, (2001) showed that flavonoids serve as anti-inflammatory, antiallergic, antithrombotic, vasoprotective, inhibits tumour, antibacterial and antifungal. Silva *et al* (1998) reported the isolation of three bioflavonoids with significant cytotoxicity against a panel of cancer cells. Isolation of flavonoids with potential cancer chemopreventive properties have also been demonstrated by bioassay guided fractionation of the active extract of *Chamenthradiffusa* using 2, 2- diphenyl- 1- picrylhydrazyl (DPPH) stable free radical antioxidant assay that led to the isolation of one novel and four known flavonoids, (Chang and Kinghorn, 2001). The presence of cadiac glycosides and saponins (two phytocompounds that have similar biosynthetic origin), draws attention to their being used traditionally as medicines but some saponins and cardiac glycosides have been said to be poisonous. At least a dozen of Apocynaceae are known to contain cadiac glycosides and extracts of these plants are mostly used as arrow poisons, (Evans, 2009). Also, Oleander ingestion caused many cases of poisoning worldwide, this is because *Merium oleander* contains oleanderin, a poisonous cardiac glycoside. However, some cardiac glycosides have medicinal benefits. Extracts from *Digitalis purpurea* and *Digitalis latana* have found relevance in heart related diseases and hence generally referred to as heart tonic, (Evans, 2009).

The antioxidant assay of C. *farinosa* (Table 4) using DPPH free radical revealed that antioxidant activity of the methanolic leaf extract was weak to moderate and inconsistent. At high concentration of 1000µg/ml the percentage inhibition was only 39.19% but on dilution to 250µg/ml, percentage inhibition rose to 53.43%. On further dilution to 125µg/ml inhibition dropped to 21.01%, and rose to 42.22% again when concentration reduced to 62.5µg/ml. this irregular variation is clearly pictured in figure 2. Antioxidant of ascorbic acid is smoothly changing with changing concentrations (Figure 1) and percentage inhibition at 1000µg/ml was 85.76% and at 62.5µg/ml was 43.84%. Comparing with that of Cadabafarinosa it can be said that C. farinosa shows inconsistent and moderate antioxidant capacity. Anticancer agents have often been isolated from plant extracts that previously showed antioxidant properties by the inhibition of 2, 2- diphenyl- 1- picrylhydrazyl, (DPPH) stable free radical, (Chang and Kinghorn, 2001; Haraguchi*et al*, 2001).

Although the methanolic leaf extact did not show appreciable antioxidant activity as expected, its cytotoxicity on Brine shrimp larvae was quite significant (Table 2). It showed significant percentage mortality of 84% at a concentration of 1000µg/ml. the percentage mortality reduced gradually and consistently in accordance with reducing concentration and therefore at the least concentration of 1.0µg/ml percentage mortality was 35.5%. This proves that the crude methanolic leaf extract was cytotoxic on Brine shrimp larvae, (nauplii). Brine shrimp lethality assay (BSLA) is an *in vivo* biological assay that has been used to select plants for antitumour and pesticide properties, (Vleitinck and Apers, 2001). Brine shrimp lethality assay has been reliably used to detect annonaceousacetagenins. Annonaceousacetogenins are powerful antitumour and pesticide products that are found only in the family annonaceae, (Mata *et al*, 2001).

CONCLUSION

In conclusion it can be said that the traditional use of *Cadabafarinosa* for the management of breast and skin cancer by the people of Bade in the North Eastern Nigeria is encourages that this plant may containpotential source of novel anticancer or precursors for the synthesis of more potent anticancer and other related chemicals including pesticides. Phytochemical analysis of methanolic leaf extract of *C. farinosa* showed the presence of flavonoids, terpenoids, saponins and reducing sugar. The current work has thrown more light on the medicinal potency of this medicinal plant because, for a plant to be both moderately antioxidative on DPPH stable free radical and significantly cytotoxic to Brine shrimp larvae, it means that the probability of isolating anticancer secondary metabolites is high because literature showed the isolation of anticancer phytocompounds from plant extracts using DPPH assay or Brine Shrimp Lethality Assay (BSLA).

SUGESTED AREAS FOR FURHTER STUDY

The current research is a preliminary phytochemical screening, antioxidant and Brine shrimp cytotoxicity assay of the methanolic leaf extract of *Cadabafarinosa*forsk (Capparidaceae) as a general primary screening assessment for

bioactivity. Now that the extract have shown to be bioactive, there is need to further purify the extract using bioactivity guided fractionation or any other means in order to find out which solvent is the best extractant for the bioactive component(s) and possibly isolate the bioactive secondary metabolite(s) for characterization and structure elucidation of the compound(s) responsible for the stated bioactivity.

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