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Bioactivity guided isolation and characterization of anti-cancer compounds from the Stem of *Musanga cecropioides* R. Br. Ex Tedlie (Urticaceae)

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Abstract
Previous studies on the stem bark of *Musanga cecropioides* showed its probable anti-cancer properties, to buttress this claims, we aimed to isolate the specific constituent(s) responsible for such activities using bioactivity guided isolation. Vacuum liquid chromatography and other chromatographic evaluations of the aqueous fraction led to the isolation of two compounds one of which was tested on AU565 breast cancer cell line. The isolated compounds were subjected to mass spectrometry on JEOL JMS 600H-1 on electron impact EI+ ionization mode and NMR (^{1}H NMR and ^{13}C NMR) were carried out on Brucker Avance 600 MHz Spectrometer at frequencies of 400 and 800 MHz respectively in DMSO to determine their respective identities. One of the isolated compound (Catechin) and the aqueous fraction showed higher anti-cancer effects than the other fractions with 20.86 and 28% cytotoxicity on AU565 cancer cell lines at 50 µg/mL. The isolated compound was identified as Catechin. The results showed that Catechin is one of the anti-cancer constituents of *M. cecropioides*.

Keywords: *Musanga cecropioides*, bioassay guided isolation, AU565 cancer cell lines, Catechin

Introduction
Plants constitute a repository of unique bioactive components which are of potential use in many applications other than in medicine [1]. Botanists describe herb as a little, seed-bearing plant with fleshy, instead of woody part.
The use of plant as resources in addressing not only food but also healthcare challenges has been part of human existence over time which transcends all social, economic, religious, and other strata of human life.
Biological activity of medicinal plants varies widely, depending on the type of plant, plant parts, geographic location and solvent used in extraction. Plants have numerous bioactive constituents like tannins, alkaloids, steroids, glycoside, fixed oils, phenols e.t.c that are contained in different parts of the plant. The therapeutic effects of plant typically occurred from one or combination of these bioactive constituents [2, 3].
As it was in the past, medicinal plants are being applied in challenging old and newly emerging ailments threatening the well-being and existence of man on earth. Such diseases include the communicable ones caused by microbes, non-communicable and life threatening ones like diabetes, hypertension, and various forms of cancers to mention a few.
M. cecropioides is a medicinal plant reputed for its application in the management of many ailments in ethnomedicine. As a plant member of Urticaceae which contains some plants with reported anti-cancer properties as documented in literature, such plants include *Broussonetia papyrifera, Myrianthus arboreus, Limonia crenulata, Phyllagathis cavaleriei* [4-7].
We have previously reported the probable anti-cancer activities of the stem bark and thus isolating the phytoconstituents responsible for the anti-cancer activities is vital to broaden the spectra of its medicinal importance.

Materials and Methods
Plant materials
Plants collection and Identification and processing
The stem bark of *M. cecropioides* was obtained within Ewu community, Esan-central LGA, Edo state, Nigeria around May, 2016. The collected plant was identified and authenticated by Mr. E.A Emmanuel (of Professor J.C. Okafor Herbarium, Pax Herbals) and voucher specimen compared with Herbarium sample in the Department of Pharmacognosy, University of Benin, Benin city.

The bark was cut into smaller pieces and air-dried at room temperature for 14 days. Thereafter, the cut pieces were transferred into an oven maintained at 50 °C for an additional 4 hours before pulverization into powder form using an electrical miller (Chris Norris, England). The powdered material was stored in air-sealed container for further use

Partitioning of the Crude aqueous extract

About 100 g of the crude aqueous extract was re-dissolved in water and partitioned exhaustively with chloroform (200 mL ×4) in a separating funnel. The lower chloroform layer was collected followed by the aqueous fraction. This was repeated until a clear lower layer was obtained. The aqueous and the chloroform fractions were concentrated on water bath and their respective yields noted.

Biological activities

Growth inhibitory effects of fractions and isolates on durra (*Sorghum bicolor*) radicle length and cytotoxic effects on tadpoles (*Raniceps ranninus*) were done consistent with Ayinde and Agbakwuru [8]. While cytotoxicity effects of fractions and isolates on AU565 carcinoma cell lines was done consistent with van Meerloo *et al*. [9] method.

Chromatographic separation and isolation

The aqueous fraction (47.5 g) was subjected to Vacuum Liquid Chromatographic (VLC) separation using air pump, Sinter glass and Buchner funnel. The fraction adsorbed on colloid (60-90/µm mesh) was eluted with 100% chloroform, followed by increasing concentrations of ester, methanol and later water to yield eight fractions which were later combined into five supported the observed activities and TLC profiling; A (< 5 mg), B (2.05 g), C (5. 11 g), D (5.93 g) and E (6.88 g) respectively.

Further cytotoxicity/anti-proliferation and TLC screening resulted combination of fractions C and D (CD = 7.05 g). This was further subjected to a different VLC using ester within the increasing concentrations of methanol which resulted to four fractions CD1 (2.05 g), CD2 (1.4195 g), CD3 (1.0037 g), CD4 (0.6522 g) and were separately subjected to cytotoxic evaluations at 5 and 10 mg/mL and anti-proliferative evaluations at 2 and 5mg/mL respectively.

Haven juxtaposed the biological activities results, fraction CD1 (2.05 g) was subjected to chromatography using colloid (60-120 µm mesh).

Elution was through with chloroform, ester and methanol in increasing order of polarity. Forty seven (47) fractions were collected and bulked together on the idea of comparable TLC profiles in ester - methanol (2:3). Fractions 16-29 were pooled together and subjected to further purification using chromatography full of sephadex LH20 where fractions 10-18 were combined to yield pure isolate. The isolate was developed in thin layer chromatography of colloid GF254 using ester - methanol (2:3) and later sprayed with 10% H2SO4 in methanol followed by heating at 110ºC for five minutes. The procedure yielded one compound coded TX1 (11.6 mg).

Characterization and Structure Elucidation of Isolated Compounds

The isolated compounds were subjected to mass spectrometry on JEOL JMS 600H-1 on electron impact EI+ ionization mode. The 1H NMR and 13C NMR were administered on Brucker Avance 600 MHz Spectrometer at frequencies of 400 and 800 MHz respectively in DMSO.

Statistical analysis

All data were expressed as mean ± SEM (standard error of mean) and n represents the number of treatment used. Where applicable, the data were compared using one-way analysis of variance (ANOVA), Graph pad Instant ® version 2.05a software (UK). The level of significance was set at $P < 0.05$.

Results

Results of the cytotoxic effects of the VLC fractions and sub-fractions of the aqueous fractions on tadpoles

The vlc fractions B, C, D and E produced concentration dependent cytotoxic effect with highest effect from fraction C at concentrations of 100 µg/mL and 200 µg/mL (83.3 ± 0.67 and 100 ± 0% mortality), followed by B at 200 µg/mL (63.3 ± 1.86% mortality) against control with no recorded mortality (fig. 1a). Fraction C and D were combined and another VLC was done to yield CD1, CD2, CD3, and CD4. The results of those sub-fractions showed that the fraction CD1 gave the very best cytotoxic effect of 76.76 ± 0.88 and 93.3 ± 0.67% mortality at concentrations of 100 and 200 µg/mL respectively (fig. 1b)

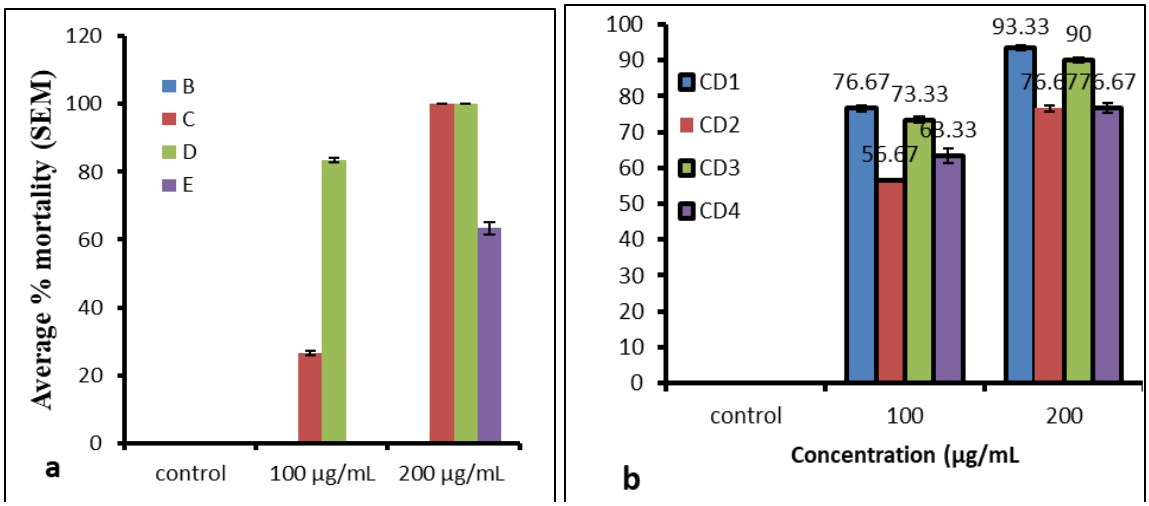


Fig 1: Cytotoxicity effects of the VLC fractions (B, C, D and E)a and sub-fractions (CD1, CD2, CD3, CD4)b of the aqueous fractions on tadpoles. Values are mean ± S.E.M, n= 10

Growth inhibitory effects of the VLC fractions and sub-fractions of the aqueous fractions on radicle length

The results of the VLC fractions showed that the bulked fraction D at concentrations of 5 and 10 mg/mL produced the very best inhibitory effects at both concentrations. While the control seeds showed average radicle length of 5.05 ± 0.17 mm in 24 hours, seeds treated with 5 and 10 mg/mL of the fraction D showed average length of 1.37 ± 0.17 and 0.43 ± 0.06 mm respectively. Also, in 96 hours, the control seeds showed average radicle length of 63.36 ± 0.42 whereas, seeds treated with 5 and 10 mg/mL of the fraction D showed average length of 14.9 ± 1.4 and 7.7 ± 1.28 mm respectively (Fig. 2).

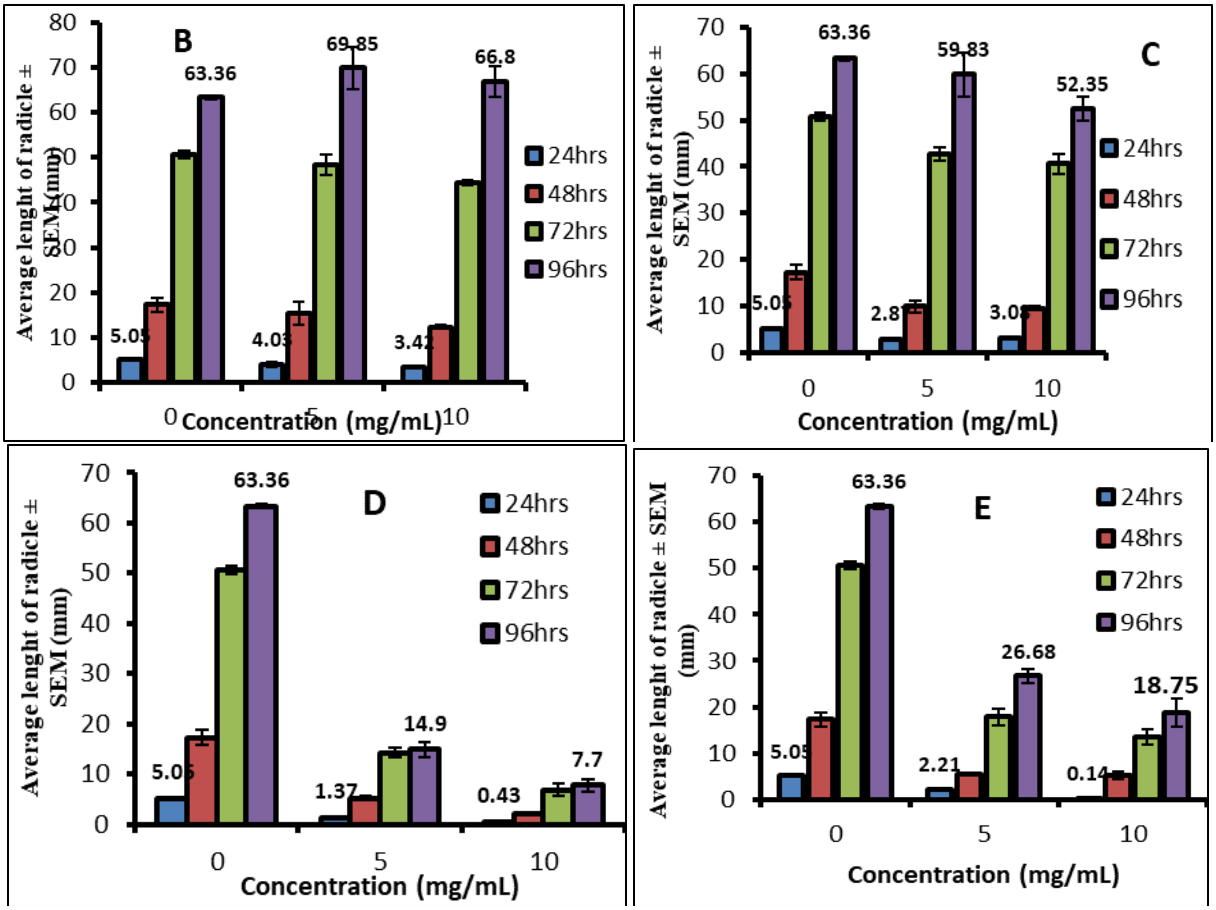
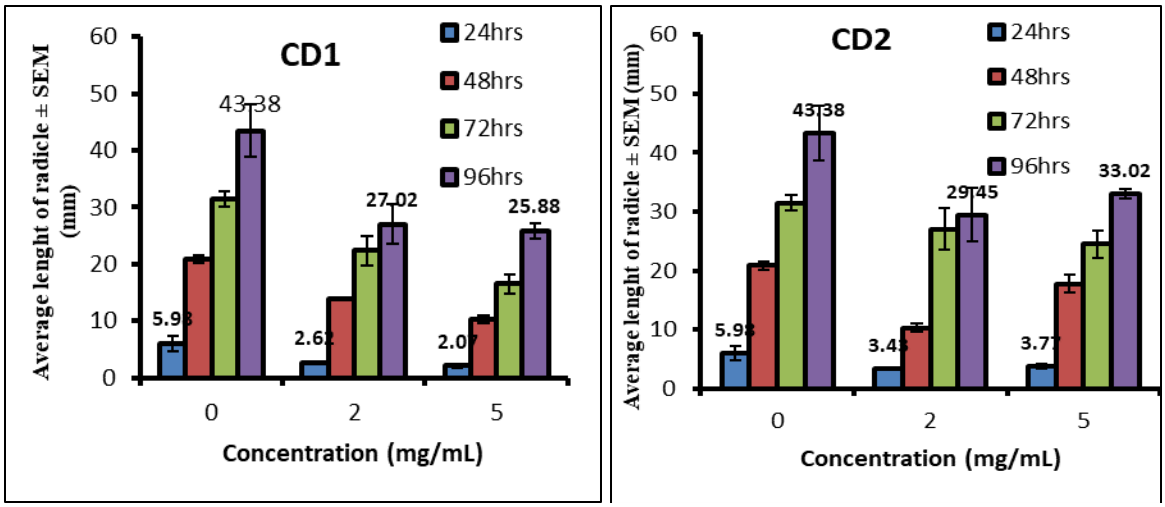


Fig 2: Growth inhibitory effect of the vlc fraction 'B, C, D, E' of the aqueous fractions on guinea corn radicle length at 24, 48, 72 and 96 hours. Values are mean ± S.E.M, n= 20

Growth inhibitory effects of the sub-fractions of the CD fraction on radicle length

Of the four (4) VLC fractions (CD1, CD2, CD3 and CD4) obtained, the results showed that the fraction CD1 at concentrations of 5 and 10 mg/mL produced the very best growth inhibitory effect. While the control seeds showed average radicle length of 5.98± 1.26 in 24 hours, seeds treated with 5 and 10 mg/mL of the fraction CD1 showed average length of two. 62 ± 0.18 and 2.07 ± 0.38. Also, in 96 hours, the control seeds showed average radicle length of 43.38 ± 4.63 whereas, seeds treated with 5 and 10 mg/mL of the fraction CD1 showed average length of 27.02 ± 3.55 and 25.88 ± 1.37 respectively (Fig. 3).



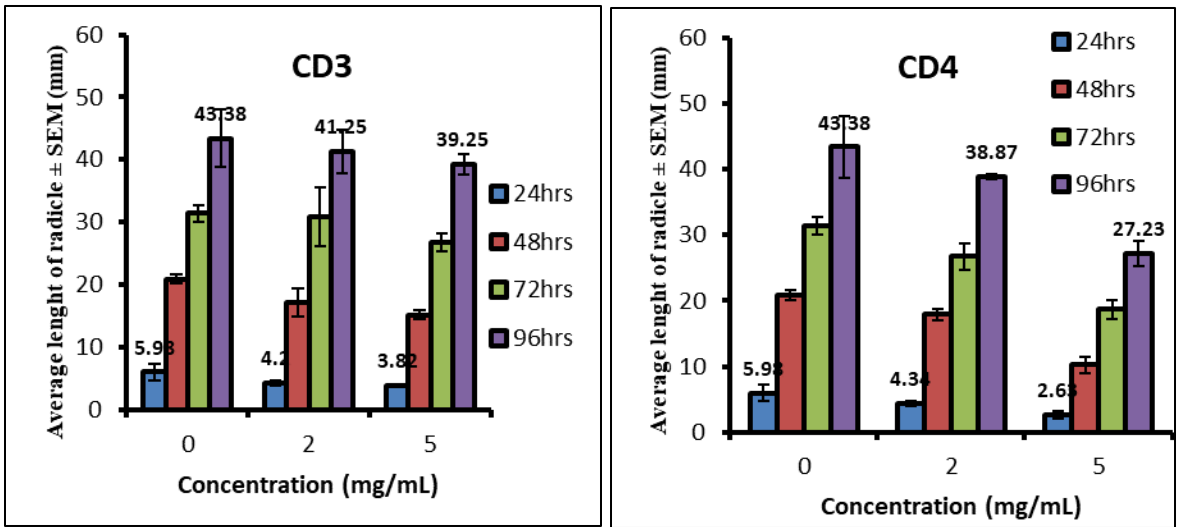


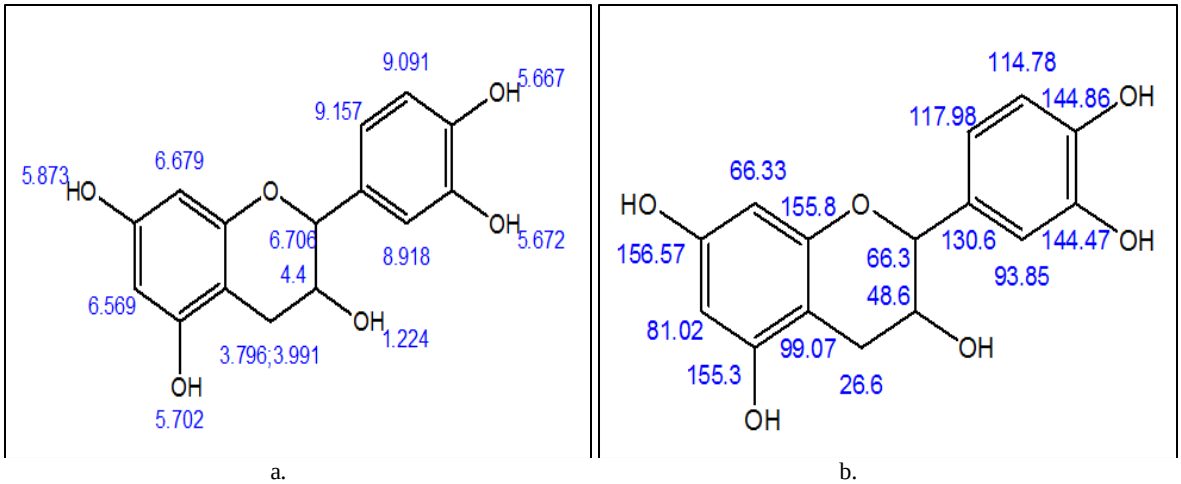
Fig 3: Growth inhibitory effect of the sub-fractions 'CD1, CD2, CD3, CD4' of the aqueous fractions on guinea corn radicle length at 24, 48, 72 and 96 hours. Values are mean ± S.E.M, n= 20.

Compound (TX1) was obtained as yellow amorphous solid. The ESI-MS showed a peak at m/z 291 [M+H]+ like the formula C15H14O6. The calculated 9 degrees of unsaturation were attributed to 6 C=C aromatics bonds and three rings with two aromatic rings. ^{1}H NMR spectrum showed the following chemical shifts on ^{1}H NMR; 9.157, 9.091, 5.667, 5.672, 8.918, 8.918, 3.796, 3.991, 5.702, 6.569, 6.569, 6.569, 6.569 and 6.569 and assig1H NMR spectrum showed the subsequent chemical shifts on 1H NMR; 9.157, 9.091, 5.667, 5.672, 8.918, 8.918, 3.796, 3.991, 5.702, 6.569, 6.569, 6.569, 6.569 and 6.569 and assigned as depicted below (figure 1a).

The 13C NMR spectrum revealed the presence of 15 carbons signals, among which: one methylene, seven methine and 7 quaternary carbons. Assignement were done as follows: δ = C-1 (155.8), C-2 (66.33), C-3 (156.57), C-4 (81.02), C-5 (155.3), C-6 (99.07), C-7 (26.6), C-8 (48.6), C-9 (66.3), C-10 (130.6), C-11 (93.85), C-12 (144.47), C-13 (144.86), C-14 (114.78), C-15 (117.98) as depicted below (1b).

Thus, compound TX1 was identified as 2H-1-Benzopyran-3,5,7-triol,2-(3,4-dihydroxyphenyl)-3,4-dihydro-,(2R-Cis)-[Catechin]. Its physical and spectral data were compared and located consistent to those reported by literature [10-13].

The Catechin is isolated here for the primary time from the stem bark of *M. cecropioides* (Urticaceae). But, it had been previously isolated from the leaves of an equivalent plant.



a. b.

Fig 4: Chemical shifts assignment for ^{1}HNMR (a) and ^{13}C NMR (b) of 2H-1-Benzopyran-3,5,7-triol,2-(3,4-dihydroxyphenyl)-3,4-dihydro-,(2R-Cis)-

Table 1: Results of fractions and compound TX1 on AU565 breast cancer cell line

Sample (50 µg/mL)	% Inhibition
CD1	28.86
CD2	-11.35
CD3	-8.01
CD4	-7.93
TX1	20.86

Effects of Fractions and compound TX1 on AU565 breast cancer cell lines

Discussion

Preliminary biological activities of the fractions of the secondary VLC showed remarkable cytotoxic activities with percentage mortality of CD1 fraction being the very best with 76.67 ± 0.89% and 93.33 ± 0.67% at 100 and 200 µg/mL respectively. Similar trend was obtained for anti-proliferative effect. While the control seeds showed average radicle length of 5.98 ± 1.26 mm in 24 hours, test seeds with 5 mg/mL of the fraction CD1 showed average length of two. 62 ± 0.18 mm yielding percentage reduction of 66.6%, CD2 (37%),

CD3 (36.1%) and CD4 (56%). Also, in 96 hours, the control seeds showed average radicle length of 43.38 ± 4.63 mm whereas, test seeds with 5 mg/mL of the fraction CD1 showed average length of 27.02 ± 3.55 mm yielding percentage reduction of 59.7%, CD2 (23.9%), CD3 (9.5%) and CD4 (37.2%) respectively. These observed activities informed the selection of fraction CD1 for further separation and isolation.

The structural elucidation of the isolated compounds were done by means of spectroscopic methods, the foremost important data were gained from NMR and mass spectrometry. Of the chromatography isolates after spectroscopy, TX1 isolated as white crystal was identified as pure compound; 2H-1-Benzopyran-3,5,7-triol,2-(3,4- dihydroxyphenyl)-3,4-dihydro-,(2R-Cis)-, also called Catechin.

However, the fractions of the secondary VLC and the isolated catechin from CD1 were subjected to cytotoxic activities against carcinoma cell line (AU565), isolate TX1 gave the very best cytotoxic effect of 20.86% followed by fraction CD2 > CD3> CD4 with -7.93 > -8.01 > -11.35% respectively. This study present for the very first time as known, the isolation of catechin from the stem bark of *M. cecropiodes*.

This compound and the gallic acid conjugates are ubiquitous constituents of vascular plants, and frequent components of traditional herbal remedies, like *Uncaria rhynchophylla*. Its two isomers are often found as vinifera grapes, *Theobroma cacao* and *Thea sinensis* phyto-constituents [14].

Among other reported pharmacological activities of Catechin is antimutagenic activities which was reported by Hayakawa *et al*. [15]. Phenolics are well known for their free radical scavenging activities which feature a direct modulatory and low affinity interference with proteins mediating cell signaling in necrobiosis [16].

Conclusion

This current study has further justified the utilization of bench-top assay methods in anti-cancer evaluation for medicinal plants. The extract and fractions were also screened *in vitro* against AU565 breast cancer cell line using MTT assay. All the tested extracts were observed to possess significant anti-proliferative/cytotoxicity properties. The aqueous extract was investigated through multistep chromatographic methods (VLC, Column chromatography and TLC) following bioactive assays (anti-proliferatory and cytotoxicity). A compound was isolated and the structure was elucidated via Nuclear magnetic resonance (NMR) and mass spectrophotometric. The biological assay on AU565 breast cancer cell lines showed that the isolated compound (Catechin) has anti-cancer activities and presented the isolation of this compound from the stem bark of *M. cecropioides* for the very first time. These results have added to the therapeutic spectrum of the plant.

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