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# Characterization of Flavonoids from *Combretum indicum* L. Growing in Egypt as Antioxidant and Antitumor Agents

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**Abstract**

**I**n developing countries, about 60-90% of their population depends on herbal extract therapy. Therefore, studies are being intensified on treatment with natural products for maintaining human health. The current work aims to characterize the bioactive metabolites by LC- ESI-MS/MS from aqueous methanol extract (AME) of *Combretumindicum* aerial parts as well as their potential antioxidant and antitumor activity. Plant material was crushed to a fine powder, then extracted with 70*%* ***(***Aq) $CH_3OH$ by soaking at room temperature (Macération). The chemical profiling of the plant was performed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) in negative mode (-ve). Fifty nine metabolites were qualitatively identified for the first time from the plant based on their retention times and fragment ions. *CombretumIndicum* aerial parts were tested for antioxidant activity using the DPPH, reducing power, ABTS, FRAP, and ion metal chelating assays. On Ehrlich ascites carcinoma cells, the entire extract was tested for its ability to inhibit cell viability %. The total extract had a reasonable impact on the viability of Ehrlisch ascites carcinoma cells at all tested concentrations in dose dependent manner. High concentration of the examined extract achieved cytotoxic effect convergent to that obtained by the commonly used drug vincristine. The results of the whole extract of *Combretumindicum* aerial parts conclusively demonstrate that this plant can be considered potential antioxidant and antitumor agents, which supports its use in complementary treatments.

***Keywords*****:** *Combretumindicum L*- HPLC-ESI-MS/MS-Antioxidant activity - Antitumor activity - Ehrlich ascites carcinoma.

## 1. Introduction

Combretaceae is a big plant family with over 20 genera and 600 species. It founds across the tropics of the world with a wide of variety in Africa and Asia [1]. The *Combretum* genus is the largest and most popular in the Combretaceae family. It comprises about 200-300 species, reported to have many biological effects as antibacterial, antioxidant, antimalarial, anti-inflammatory, and cytotoxicity against tumor cells[2].The genus *Combretum*in African traditional medicine, are well-known to treat a wide range of ailments and diseases, includes fever and microbiological infections, as well as heart and worm problems, mental health issues, and bites from scorpions and snakes. There are medicinal uses for all plant parts of the *Combretum*species, including occasionally the fruits [3]. Also *Combretum* species have edible kernels [4]. In traditional medicine, some *Combretum* species are widely used as diuretics and to treat inflammatory diseases, infections, diabetes, malaria, bleeding, diarrhea, and digestive problems.Many secondary metabolites, including flavonoids, triterpenes, lignans, phenanthrenes and stilbenoids were discovered in the *Combretum* genus through phytochemical investigation of *Combretumindicum* CIA (Syn.*QuisqualisIndica*L. also recognized as Rangoon Creeper) is one of the most popular species of *Combretum*growing in Egypt. In West Africa, ripe seeds are said to be sweet and enjoyable to eat if the seed coat is removed. Although, the flowers have no flavour, they are also reported as edible and can be added to salads to add color. very young shoots are eaten raw or steamed in Indonesia due to its therapeutic and nutritional benefits. The medicine "santonina" is replaced in China with seeds of the CIA plant based on its active ingredients especially (phenolics& flavonoids) and biological safety [5-7]. The search for biologically active antitumor compounds is still on going because

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many patients with tumours develop resistance to antitumor agents during treatment, in addition to the undesired side effects of these antitumor agents. Plants provide a great possibility for finding fresh sources of antitumor agents with new action mechanism and negligible toxicological effects. Therefore, the aim of this study was to investigate CIA extract to identify the antioxidant and antitumor components using modern and rapid HPLC-ESI-MS/MS-technique. Fifty nine metabolites were detected by HPLC-ESI-MS/MS which are extracted to be responsible for antioxidant and antitumor effects of CIA extract.

## 2. Materials and Methods

### 2.1. Plant-based materials

During the flowering season, February to May 2020, a complete plant sample was taken from National Research Centre (NRC) garden (Dokki, Cairo, Egypt).The plant species was identified by Dr. M. EL-Gibaly, Lecturer of Taxonomy and Consultant for Central Administration of Plantation and Environment. Avoucher sample (No: B79), was deposited at chemistry of tanning materials and leather technology department .National Research Centre, Dokki, Cairo, Egypt. The entire sample was homogenised into a fine powder and left for extraction after being air-dried for 15 days in the shade.

### 2.2. Chemicals and drugs

Di-ionized water (DI-$H_2O$) is further purified using the Milli-Q Plus water purification equipment from Millipore Ltd. in Bedford, Massachusetts, as well as analytical-grade methanol and dimethyl sulfoxide HPLC from Merck in Darmstadt, Germany. The following substances were bought from Sigma Chemical Co.: 2,2-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), butylatedhydroxytoluene (BHT), $FeCl_3$, Trolox, 2, 4, 6-tripyridyl-s-triazine (TPTZ) (St. Louis, MO, USA). The solvents and all other compounds were of analytical grade. The 1640 medium for Roswell Park Memorial Institute (RPMI) was bought from Sigma Chemical Company (St. Louis, MO, USA). All additional reagents and chemicals were of analytical reagent quality.

### 2.3. Equipment and Conditions

#### 2.3.1. HPLC/ESI-MS/MS analysis

The analysis of the sample was performed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) with an ExionLC AC system for separation and SCIEXTriple Quad 5500+ MS/MS system equipped with an electrospray ionization (ESI) for detection. The separation was performed with aAscentis® C18 Column (4.6×150 mm, 3µm). The mobile phases were consisted of two eluents, A: 0.1% formic acid; B: acetonitrile (LC grade)**.** The mobile phase gradient was programmed as follows: 10% B at 0-2 min, 10-90% B from 2-30 min, 90% B from 30-36 min, 10% at 36.1, 10% from 36.1-40 min. The flow rate was 0.7 ml/min and the injection volume was 10µl. For MS/MS analysis, negative ionization mode was applied with a scan (EMS-IDA-EPI) from 100 to 1000 Da for $MS^1$ with the following parameters: curtain gas: 25 psi; IonSpray voltage:-4500; source temperature: 500°C; ion source gas 1 & 2were 45 psi and from 50 to 1000 Da for $MS^2$ with a declustering potential: -80; collision energy: -35; collision energy spread. The identification of constituents was performed using MS-DIAL software version 4.70 and Fiehn HILIC library.

### 2.4. Extraction Methods

A total of (50 g) of air-dried powder of the aerial parts of CIA was extracted with 70*% (*Aq) $CH_3OH$ (2L) by soaking at room temperature (Macération). A rotary evaporator was used to evaporate the solvent under vacuum at 50°C. Then the residue (dry) was sequentially defatted and desalted by $CHCl_3$ and $C_2H_5OH$, respectively, by warming under reflux conditions. Thereafter, the residue was taken in methanol, affording a methanol-soluble portion (MSP) that yielded AME of 15 g.

### 2.5. Investigation of antioxidant activity

The detailed methodologies of DPPH, ABTS radical scavenging activity, reducing power ability, ferric reducing power (FRAP), and Metal chelating activity assays were reported in our previous papers **[8, 9]**.

### 2.6. *In vitro* anticancer activity

Cytotoxicity and cell viability test were measured microscopically according to Ahmed et al., 2023&Haroun and Taie, 2015, by viable cell counting **[8,10]**. The optimal concentration of AME from CIA (25, 50, 100, 150, and 200 µg/ml) was estimated. The viability percentage of tumor cells was measured after incubation with the different concentrations of AME from CIA. A suspension of the tumor cells was attained from peritoneal cavities of tumor-bearing mice and then diluted with phosphate buffered saline (pH 7) so that the final preparation comprised $2.5x10^5$ cells/µl. In a set of sterile test tubes, aliquots (0.1 µl / tube) of the cell preparation were distributed followed by addition of aliquots (0.8µl / tube) of phosphate buffered saline (PBS).

The investigated concentrations of CIA (Dissolved in phosphate-buffered saline) were then applied to the tubes in aliquots (0.1 µl / tube). The tubes were incubated at 37°C for 2h under 5%$CO_2$, then the tubes were centrifuged at 1000 rpm for 5 min and separated cells were suspended in saline. For each examined concentration and control, a new clean, dry small test tube was used and 10µl of cells suspension, 80 µl saline and 10 µl trypan blue were added and mixed, and then the number of living cells was calculated using a hemocytometer slide. Survived cells appeared as unstained bodies while non-viable cells stained blue. Vincristine was used as a standard drug (100µg/ml).

## 3. Results and Discussion

### 3.1. Qualitative characterization of active constituents in AME of *C. indicum* areal parts by HPLC/ESI-MS/MS analysis

In the AME of CIA fifty nine metabolites belonged to various classes of natural products were tentatively identified by LC- MS/MS analysis. Total ion (TIC) and base peak (BPC) chromatograms **(Figure1)**are based on structural information from HPLC/ESI-MS/MS data, particularly [M-H]- ions. The study of the output data, including Rt-values, molecular formulae, molecular ion peaks [M-H], selected fragment ions, and their comparison with previously published data was necessary to identify the components in the extract (PeakView 1.2, MSDIAL 4.90 and or Analyst TF 1.7.1 softwares). **Figures (3-6)**showed $MS^2$ spectra of some identified components listed in **Table1**. Accurate mass measurements, main product ions, retention time and formula for all compounds are summarized in **Table 1**.These compounds numbered and arranged according to their classes and retention time. The compounds' structures were drawn and classified by Chem Bio Draw Ultra 16.0 as shown in **Figure2**. The detected compounds included10 phenolic acids, 29 flavonols, 9 flavones, 4 tannins, 2 flavanones, 2coumarins and one another organic compound. There were some observed peaks that could not be categorized or identified, however they were small in comparison to the overall number of identifications. The class with the highest abundance was flavonols (Quercetin, isorhamnetin, kaempferol, myricetin and their derivatives). In this work all of the separated compounds were tentatively identified and detected for the first time in Egyptian CIA. The presence of different types of flavonols derivatives (methylated, and glycosylated) in such abundance may contribute to the antitumor effect of the CIA.

#### 3.1.1. Tentative identification of phenolic acids

The loss of a $CO_2$ (44 Da) from the carboxylic acid group is a key characteristic of the fragmentation patterns produced by phenolic acids. The negative ESI-MS spectrum for compounds **2, 4-6** and **9** showed deprotonated ions at m/z 179.00, 167.06 , 136.93, 193.05 & 163.10 and product ions at m/z 135.07, 123.01, 108.98, 139.03&116.99 respectively, due to the cleavage of $CO_2$. Metabolites 3, 10, 7 and 8 displayed characteristic fragments for phenolic acids glycosides and chlorogenic acid at m/z179.11[M-H-Hex(162)] &135.03[M-H-(Hex(162)+$CO_2$)] for Caffeic acid hexoside, 163.04[M-H-Gluc (176)] for *(E)-p*-coumaric acid glucuronide, 339.20[M-H-Gluc(176)] &163.19[M-H-di Gluc (352)] for *(E)-p*-coumaric acid di glucuronide and 163.18[M-H- (190)] & 177.13[M-H-Gluc (176)] for chlorogenic acid **[11]**.

#### 3.1.2. Tentative identification of flavonoids

The chemical structures of individual flavonoids were determined by fragment pattern analysis, in which flavonoid glycosides such as glucose, rhamnose, pentose, glucuronic acid and neohesperidoside (m/z 162, 146, 132, 176 and 308) were cut from their structures. All flavonoids were detected to be glycosides with one or more sugar moieties. There were both *O*-glycosylated and *C*-glycosylated flavonoids found. The $MS^2$ fragmentation pattern makes it simple to distinguish between these two flavonoid groups. The primary cleavages occur at the bonds of the sugar because the carbon-carbon bond of *C*-glycosylated flavonoids is resistant to rupture. *O*-glycosylated flavonoids, however, easily lose the sugar moieties due to neutral losses **[12]**.

##### 3.1.2.1. Tentative identification of kaempferol derivatives

Various derivatives of kaempferol were detected in AME of CIA aerial parts. All the kaempferol glycosides detected were of the type *O*-glycosylated and methylated flavonoids. The presence of the characteristic product ions285& 284 and neutral loss of [M-H- Hex(162)], [M-H- pentose (132)], [M-H-rham (146)], [M-H- di Hex(324)], [M-H-(rham (146)+Hex(162)], and [M-H-(Gluc (176)+Hex(162)], indicated peaks **19**, **20**, **25**, **28**, **32**, **33** and **34** were originated from kaempferol or lutiolinaglycone. $MS^2$ spectrum was used to confirm the aglycone and provide additional information. The aglycone was identified as kaempferol as a result of the differentiated product ions at m/z 255 and 227. According to these characteristic fragments the compounds were tentatively identified as Kaempferol7-*O*-α-L-rhamnoside, Kaempferol 4´- di-*O*-hexoside, Kaempferol7-*O*-neohesperidoside, Kaempfero3-*O*-glucuronide7-*O*-hexoside, Kaempferol3-*O*-pentoside, Kaempferol3 -*O*-hexoside, respectively **[13]**.

**The 3-OH glycosylation site** was confirmed by the appearance of two high abundance fragments at m/z 285 & 284 and the intensity of m/z284 is higher than that of m/z 285**(Figure 3)**. The substitution **of 7-OH** glycosylation was proved by the predominant ion at m/z 285 coupled with the weak

ion at m/z 284. The substituent position at **4´-OH** resulted from the absence of the ion at m/z 284 that coupled with the abundance ion at m/z 285**(Figure 4)**.

Based on the precursor ion $[M-H]^-$ at m/z 593.01at ($R_t$, 8.905), characteristic product ions at m/z 447.06[M-H-146]-, 307.20, two main fragments at m/z 284.98 & 284.04[M-H-(146;coumaroyl+ 162;hexoside)]$^-$ and the loss of (-308) amu suggested the *p*- coumaroyl and hexose linked at the same position of the aglycone. The intensity of 284.98[M-H - 308]$^-$ is higher than 284.04 [M-H-308]$^-$ indicated the substitution of *p*-coumaroylhexoside at **5-OH positions**. These results porposed compound **34** as kaempferol 5-*O* (6-*P*-coumaroyl hexoside. According to Karioti *et al*., Felipe et al. and Zhuan-Hong et al., additional confirmation of 34 shown by distinctive product ion at m/z 307[M - H- Kaempferol (285)] was due to the loss of p-coumaroyl group rather than rhamnose moiety **(Figure5)[14-16]**.

The MS-MS showed $[M-H]^-$ at m/z 298.9, two predominant ions at m/z 284.48 [M-H-$CH_3$], 282.9604 [M-H- $CH_3$].The high intensity of the main ion at 284.48 proved the ($CH_3$) group linked at the position7-OH so compound **39** characterized asKaempferide (7-methoxy kaempferol) or rhamnocitrin**[13]**.

**3.1.2.2. Tentative identification of quercetin derivatives**

On the basis of MS/MS spectral data all compounds **11**, **14-16**, **23**, **24**, **26**, **30**, **31**, **38** produced the deprotonated aglycone fragment at m/z 301 as illustrated in **Table 1**. The appearance of the main ion in these compounds at m/z301coupled with the weak fragment 300 and typical ions at m/z 255.00, 179.00, 271.00 and 151suggested the aglycone of quercetin. Using the mass spectral information Compounds**11, 16, 23, 24** were tentatively identified as quercetin3- *O*-α-L-rhamnoside, quercetin3- *O*-arabinside, quercetin3- *O*-hexoside,quercetin3- *O*-pentoside due to loss of sugar moiety. The loss of 324amu(162+162) indicated that the presence of two hexoses linked at the same position of aglycone so compound **14** was detected as quercetin7- *O*- di Hexoside due to the high intensity of m/z 301[M-H-324]- than 300[M-H-324]$^-$ **(Figure 6)**. A hexose and a deoxyhexose were also joined at the same position of the aglycone, according to m/z 301, which corresponds to the loss of 308 amu (162 + 146). The low intensity of m/z 301[M-H-308]-than 300[M-H-308]$^-$proved the compound **30** was suggested as quercetin3- *O*-neohesperioside(rutin). By the same way compound **31** was identified tentatively as quercetin 3-*O*-hexoside7-*O*-glucuronide. Two isomers from quercetine-di methyl ether (**26 & 27)** showed the same molecular ion peak at m/z 328.90 with the same product ions at 298.91[M-H-2$CH_3$]-, 313,98[M-H-$CH_3$]-and have different retention time at (7.078&7.74) were confirmed as quercetine5, 3´di methyl ether and quercetine3', 7-di methyl ether while compound **15** was confirmed as quercetine3-*O*-hexoside 5, 3´di methyl ether **[17]**.

**3.1.2.3. Tentative identification of isorhamnetin**

In this investigation five derivatives of isorhamnetin were detected by ESI-MS/MS analysis. As indicated in **Table1**, compounds **12**, **13**, **21, 22 and 29** originated from isorhamnetinaglycone based on the characteristic deprotonated fragment at m/z 315 and product ions at m/z 299.9, 255, 285 and 271.03. Compounds **12**, **21** and **22** produced [M-H]- at m/z 446.89, 476.98 and 490.93, respectively. MS/MS spectral data of compound**12** showed two predominant ions at m/z 315.01[M-H-132]$^-$, 314.03 [M-H-132]. The intensity of [M-H–132] is higher than that of [M-H-132] suggested the substituent position of 3-OH so compound **12** confirmed as isorhamnetin3-*O*-pentoside. By the same way compounds **21&22** were tentatively identified as isorhamnetin3-*O*-hexoside and isorhamnetin3-*O*-glucuronide. The loss of di-hexose unit (324amu) proved that the di- hexose linked at the same position of aglycone and established compound **29** as isorhamnetin3-*O*-di hexoside. Compound **13** ($R_t$= 3.008, M.F $C_{28}H_{34}O_{17}$) gave a [M-H]- at m/z 597.24 and two intensive fragments at m/z 507.09[M-H- 90]-, 477.07[M-H- 120]. The loss of 120 & 90 amu indicated that a di-hexose linked as C-glycosidic linkage. The high intensity of a predominant ion at m/z 315.20[M-H- (120+ 162)] confirmed that hexose at 8-*C*-position of aglycone in A-ring. The loss of 120amu followed by loss of 162 indicated that there are two hexoses at two different positions (3-*O*-substitution and 8-*C*- position). Thus compound **13** was tentatively characterized as Isorhamentin 3-*O*-hexoside8- *C*- hexoside**[16, 18]**.

**3.1.2.4. Tentative identification of myricetin**

Two derivatives of myricetin were tentatively established based on their intensive fragment at m/z 317 which indicated that they were derived from myricetin as aglycone. Peak **17** gave [M–H]- at *m/z* 462.95 and characteristic product ions at *m/z* 317.07 [M-H-(146)], 301.03 [M-H-(146+$CH_3$)] & 271.09 was tentatively identified as myricetin3-*O*-α-Lrhamnoside. Compound **40** was tentatively established as myricetin8-*C*-hexoside due to loss of 120amu that distinct for *C*-glycosidic linkage.

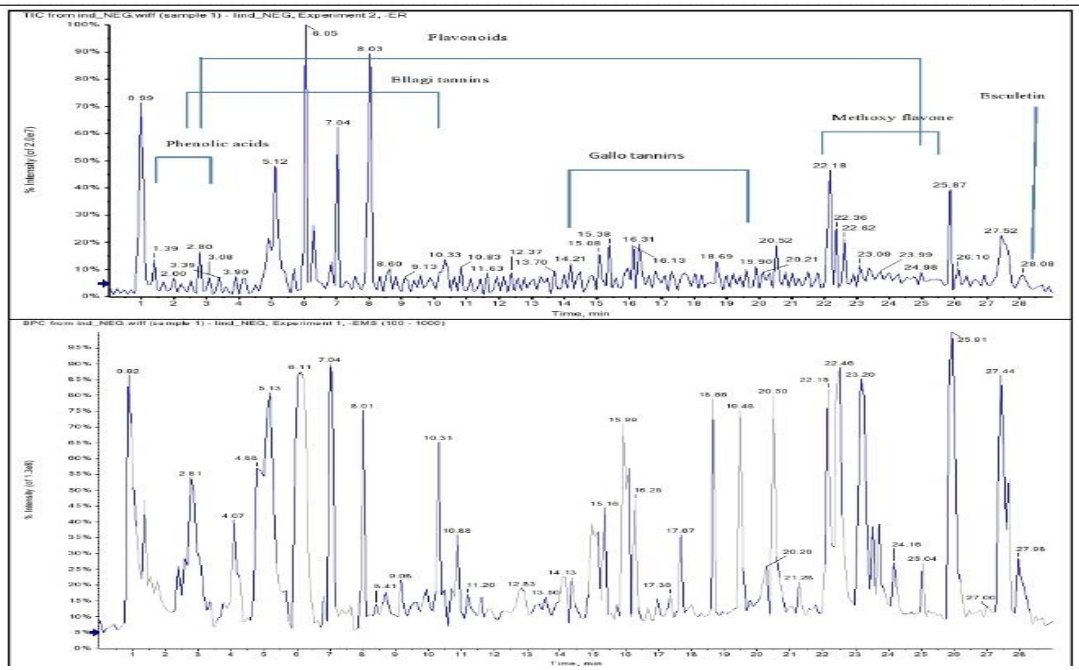



**Fig. 1. TIC and BPC chromatograms of AME from aerial parts of *C. indicum***

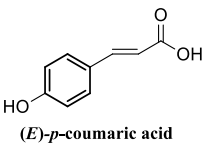
**TABLE1. Metabolites identified in AME of C. indicum aerial parts using HPLC/ESI-MS/MS in negative ionization mode (-ve)**

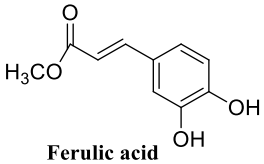
| PEAK NO | $R_T$ MIN | TENTATIVELY IDENTIFIED COMPOUNDS | CLASSES | $[M-H]^-$ M/Z | FORMULA | $MS^2$ MAJOR PRODUCT ION |
|---|---|---|---|---|---|---|
| Phenolic acids | | | | | | |
| 1 | 0.903 | Citric acid | Phenolic acid | 190.96 | $C_6H_8O_7$ | Not fragment |
| 2 | 1.067 | Caffeic acid | Phenolic acid | 179.00 | $C_9H_8O_4$ | 134.03[M-H-COOH]-, 135.07[M-H-$CO_2$]- |
| 3 | 1.611 | Caffeic acid hexoside | Phenolic acid glycosides | 340.95 | $C_{15}H_{18}O_9$ | 135.03, 179.11[M-H- hex (162)]-,153.12,161.04[M-H- (hex (162)+$H_2O$)]-, 132.96, 135.03[M-H- ( hex(162)+$CO_2$)]-, 204.97 |
| 4 | 5.416 | Vanillic acid | Phenolic acid | 167.06 | $C_8H_8O_4$ | 123.01[M-H-$CO_2$]- |
| 5 | 7.712 | Salicylic acid | Phenolic acid | 136.93 | $C_7H_6O_3$ | 108.98[M-H-CO]- |
| 6 | 8.209 | Ferulic acid(_Hydroxycinnamic acid) | Phenolic acid | 193.05 | $C_{10}H_{10}O_4$ | 134.03[M-H-($CH_3$+$CO_2$)]-, 161.04[M-H-$OCH_3$]-, 133.00, 132.00 |
| 7 | 16.897 | Chlorogenic acid | Phenolic acid | 353.16 | $C_{16}H_{18}O_9$ | 163.18[M-H- (190)]-, 177.13[M-H-Gluc (176)]- |
| 8 | 22.092 | *(E)-p*-Coumaric acid glucuronide | Phenolic acid glycosides | 339.1 | $C_{15}H_{16}O_9$ | 163.04[M-H-Gluc (176)]- |
| 9 | 22.230 | *(E)-p*-Coumaric acid | Phenolic acid | 163.10 | $C_9H_8O_3$ | 119.05, 116.99 [M-H-$CO_2$]- |
| 10 | 25.111 | *(E)-p*-Coumaric acid -di glucuronide | Phenolic acid glycosides | 515.16 | | 339.20[M-H-Gluc (176)]-163.19[M-H- di Gluc (352)]- |
| Flavonols | | | | | | |
| 11 | 2.575 | Quercetin 3-*O*-rhamnoside | Flavonol mono- *O*-glycoside | 446.96 | $C_{21}H_{20}O_{11}$ | 301.00[M-H- rham (146)]-, 300.00, 255.00, 179.00 |

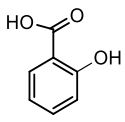
| | | | | | | |
|---|---|---|---|---|---|---|
| 12 | 2.895 | Isorhamnetin3-*O*-pentoside | Flavonol mono- *O*-glycoside | 446.89 | $C_{20}H_{18}O_{11}$ | 315.01[M-H - pentose (132)]-, 300.04[M-H -(pentose (132)+$CH_3$)]-, 301.01, 314.03 |
| 13 | 3.008 | Unknown | Flavonol di glycoside | 597.24 | $C_{28}H_{34}O_{17}$ | 507.09[M-H- 90]-, 477.07[M-H- 120]-, 315.20[M-H- (120+ Hex(162)]- |
| 14 | 3.550 | Quercetin7- di-*O*-hexoside | Flavonoldi -*O* glycoside | 624.95 | $C_{27}H_{30}O_{17}$ | 462.96[M-H- hex (162)]-,301.08[M-H- hex (162)]-, 300.10 255, 179 |
| 15 | 5.141 | Querceine3-*O*-hexoside 5, 3´di methyl ether | Methoxylatedflavonol glycoside | 490.95 | $C_{23}H_{24}O_{12}$ | 475.90[M-H-$CH_3$]- , 329.01[M-H- hex (162)]-, 313.04[M-H- ( hex (162)+$CH_3$)]-, 298.91[M-H- ( hex (162)+2$CH_3$)]- , 301.09[M-H-190]-, 300.00, 271.00, 151.03 |
| 16 | 5.244 | Quercetin 3-*O*-arabinoside | Flavonol mono- *O*-glycoside | 432.90 | $C_{20}H_{18}O_{11}$ | 300.97[M-H- (132)]-, 300.02, 255.09, 271.07 |
| 17 | 4.455 | Myricetin3-*O*- α-Lrhamnoside | Flavonol | 462.95 | | 317.07[M-H- (146)]-, 301.03[M-H-(146+$CH_3$)]-, 271.09 |
| 18 | 5.459 | Dihydrokaempferpl hexoside | Flavonol glycoside | 449.01 | $C_{21}H_{22}O_{11}$ | 286.94[M-H- hex (162)]-, 151.00 |
| 19 | 5.790 | Kaempferol3-*O*-hexoside | Flavonol mono- *O*-glycoside | 446.96 | $C_{21}H_{20}O_{11}$ | 284.98[M-H- hex (162)]-, 283.99, 255.11, 227.17 |
| 20 | 6.390 | Kaempferol3-*O*-pentose | Flavonol mono -*O* glycoside | 416.95 | $C_{20}H_{18}O_{10}$ | 284.97[M-H- pentose (132)]- 284.02, 255.08, 227.09 |
| 21 | 6.413 | Isorhamnetin3-*O*-hexoside | Flavonol mono- *O*-glycoside | 476.98 | $C_{22}H_{22}O_{12}$ | 315.04[M-H- hex(162]-, 299.07[M-H- ( hex(162)+$CH_3$)]-, 271.06 |
| 22 | 6.422 | Isorhamnetin 3-*O*-glucuronide | Flavonol mono- *O*-glycoside | 490.93 | | 314.80 |
| 23 | 6.490 | Quercetin3-*O*- hexoside | Flavonol mono- *O* -glycoside | 462.98 | $C_{21}H_{20}O_{12}$ | 301.05[M-H- hex (162)]-, 300.05 , 151.10, 179.16, 255.12, 271.09. |
| 24 | 6.523 | Quercetin3-*O*-pentoside | Flavonol mono- *O* glycoside | 432.96 | $C_{20}H_{18}O_{11}$ | 301.03[M-H- Pentose (132)]-, 300.01, 271.07, 179.04, 255 |
| 25 | 7.044 | Kaempferol7-*O*-α-L-rhamnoside | Flavonol mono- *O*-glycoside | 430.93 | $C_{21}H_{19}O_{10}$ | 284.99[M-H- rham (146)]-,255.10, 227.15, 229.17 |
| 26 | 7.078 | Querceine 5, 3´di methyl ether | Methoxylatedflavonol | 328.93 | $C_{17}H_{14}O_7$ | 313.98[M-H-$CH_3$]-, 298.91[M-H-2$CH_3$]-, 271.03 |
| 27 | 7. 74 | quercetin3',7-dimethyl(Rhamnazin) | | 328.93 | $C_{17}H_{14}O_7$ | 313.98[M-H-$CH_3$]-, 298.91[M-H-2$CH_3$]-, 271.03 |
| 28 | 8.160 | Kaempferol4´ di-*O*-hexoside | Flavonol di-*O* glycoside | 608.93 | $C_{27}H_{30}O_{16}$ | 447.10 [M-H- hex(162)]-, 285.01 [M-H- di hex(324)]-, 255.10, 227 |
| 29 | 8.246 | Isorhamnetin di-*O*-hexoside | Flavonol di-*O* glycoside | 638.92 | $C_{28}H_{32}O_{16}$ | 476.94[M-H- hex (162)]-, 315.08[M-H- di hex (324)]- |
| 30 | 8.442 | Rutin | Flavonol di-*O* glycoside | 608.99 | $C_{27}H_{30}O_{16}$ | 462.96[M-H-rham(146)]-,301.06 [M-H-(rham (146) + hex(162)]- |
| 31 | 8.624 | Quercetin 3-*O*-hexoside7-*O*-glucuronide | Flavonol di-*O* glycoside | 638.92 | $C_{27}H_{28}O_{18}$ | 476.94[M-H- hex (162)], 462.96 [M-H- Gluc (176)]-301.11[M-H-(Gluc(176)+ hex (162)]-, 300.07, 179.01 |

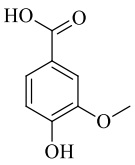
| | | | | | | |
|---|---|---|---|---|---|---|
| 32 | 8.654 | Kaempferol-7-*O*-neohesperidoside | Flavonol di-*O* glycoside | 592.99 | $C_{27}H_{30}O_{15}$ | 446.99[M-H-rham (146)]-, 285.00 [M-H-(rham (146)+ hex(162)]-, 255.21, 227.10, 283.89 |
| 33 | 8.849 | Kaempferol3-*O*-glucuronide7-*O*-hexoside | Flavonol di-*O* glycoside | 622.96 | $C_{27}H_{28}O_{17}$ | 447.06[M-H- Gluc (176)]-, 285.01[M-H- Gluc (176)- hex(162)]-, 255.09, 227.17 |
| 34 | 8.905 | Kaempferol 5-*O* -(6-*P*-coumaroyl hexoside | Flavonolglucoside derivatives | 593.01 | $C_{30}H_{26}O_{13}$ | 447.06[M-H- coumaroyl (146)]-, 307.20, 284.98[M-H – (308Coumaroylhexoside]-,284.04, 255.20 |
| 35 | 10.255 | Kaempferol | Flavonol | 285.00 | $C_{15}H_{10}O_6$ | 255.08, 227.05, 169.16, 185.06 |
| 36 | 10.837 | Myricetin | Flavonol | 317.00 | $C_{16}H_{12}O_7$ | 180.13, 248.00 |
| 37 | 11.742 | Isorhamentine | Methoxyflavonol | 314.99 | $C_{16}H_{12}O_7$ | 299.9, 255, 285, 271.03, 243 |
| 38 | 19.950 | Quercetin | Flavonol | 301.15 | $C_{15}H_{10}O_7$ | 285.18, 273.08, 257.13, 229.16, 179.04, 169.20, 151.04, 111.00, 106.97 |
| 39 | 23.007 | Kaempferide (7-*O*-methoxykaempferol) or rhamnocitrin | MethoyFlavonol | 298.9 | $C_{16}H_{12}O_6$ | 284.48 [M-H-$CH_3$]-, 282.96, 255.05, 151.07, 132.00 |
| 40 | 27.723 | Myricetin8-*C*-hexoside | Flavonol | 479.12 | | 359.16 [M-H-120]-,317.01[M-H- hex (162)]-, 151.00 |
| Flavones | | | | | | |
| 41 | 2.615 | Apigenin 7-*O*- hexoside | Flavone mono-*O*-glycosides | 430.98 | $C_{21}H_{22}O_{10}$ | 269.10[M-H- hex (162)]-, 266.00 |
| 42 | 5.543 | Luteolin 6-*C*-hexoside | Flavone mono-*C*-glycosides | 446.94 | $C_{21}H_{20}O_{11}$ | 357.02[M-H-90]-, 327.02[M-H-120]-,285.00[M-H- hex ( 162)]- |
| 43 | 8. 387 | Apigenin7-*O*-rutinoside | Flavone mono-*O*-glycosides | 577.06 | $C_{27}H_{30}O_{14}$ | 414.97[M-H- hex(162)]-, 399.08[M-H-( hex(162)+($CH_3$ of rhamnosemoity)]-, 268.98[M-H-(308)]-, 292.82, 163.09 |
| 44 | 8.601 | Diosmin | 4' Methoxy flavone-diglycoside | 607.05 | $C_{28}H_{32}O_{15}$ | 589.00, 575.07, 561.07, 501.01, 399.26, 207.12, 131.00, 147.00 |
| 45 | 9.943 | Luteolin7-*O*-hexoside | Flavone mono-*O*-glycosides | 446.93 | $C_{21}H_{20}O_{11}$ | 285.00, 284.03, 271.03 |
| 46 | 9.777 | Luteoline | Flavone | 285.00 | $C_{15}H_{10}O_6$ | 133.06, 151.03, 175.02 |
| 47 | 16.508 | Chrysoeriol (3'-methoxy derivative of luteolin) | 3'-methoxy derivative of luteolin) | 299.11 | $C_{16}H_{12}O_6$ | 284.10 |
| 48 | 24.416 | Apigenin | Flavone | 269.24 | $C_{21}H_{20}O_{10}$ | 116.94 |
| 49 | 24.832 | Apigenin 7-*O*-methoxide | Methoxy flavone | 283.22 | $C_{21}H_{20}O_{10}$ | 268.89.10[M-H-$CH_3$]-, 239.04, 135.04 |
| Hydroquinones | | | | | | |
| 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Flavanones | | | | | | |
| 51 | 51 | 51 | 51 | 51 | 51 | 51 |

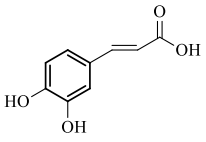
| | | | | | | |
|---|---|---|---|---|---|---|
| 52 | 52 | 52 | 52 | 52 | 52 | 52 |
| Coumarins | | | | | | |
| 53 | 28.483 | Daphnetin (7,8-dihydroxycoumarin) | Coumarin | 177.01 | $C_9H_6O_4$ | 162.04, 145.07, 133.07[M-H-COO]-, 120.95, 118.01, 117.04 |
| 54 | 28.543 | Esculetin (6,7-dihydroxycoumarin) | Coumarin | 176.91 | $C_9H_6O_4$ | 158.90[M-H-$H_2O$]-, 141.00[M-H-2$H_2O$]- 133.08[M-H-COO]- |
| Ellagi and gallo tannins | | | | | | |
| 55 | 2.276 | Ellagic acid rhamnoside | Ellagi tannins | 446.91 | $C_{20}H_{16}O_{12}$ | 300.03, 300.98[M-H –rham(146)]-, 257.06 |
| 56 | 10.20 | Ellagic acid | Tannins | 301.01 | $C_{14}H_6O_6$ | 283.99[M-H -$H_2O$], 200.00 [M-H-($H_2O$+3CO]- 145.00 , 229.07, 283.93, 255.25 |
| 57 | 11.488 | Ellagic acid hexoside | Ellagi tannins | 462.97 | $C_{20}H_{16}O_{13}$ | 301.03[M-H-hex(162)]- |
| 58 | 19.924 | Gallic acid | Phenolic | 152.05 | $C_7H_6O_5$ | 137.04, 135.02, 121.03, 122.00 |
| Organic compounds | | | | | | |
| 59 | 17.619 | Para nitrophenol | | 138.02 | | 108.00 |
| Hex : glucose/galactosemoietyl; Gluc: glucuronide moiety: rham: rhamnoside moiety | | | | | | |

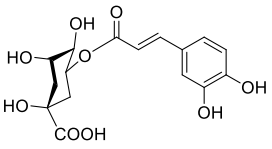

(*E*)-*p*-coumaric acid

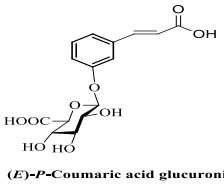

Ferulic acid

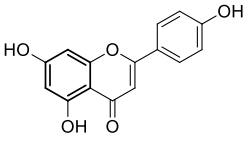

Salicylic acid

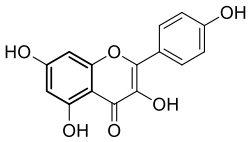

Vanillic acid

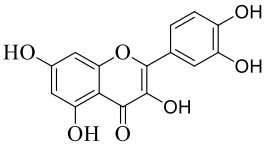

*E*-Caffeic acid

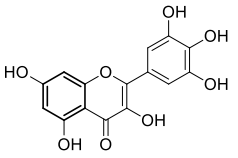

Chlorogenic acid

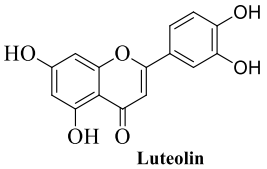

(*E*)-*P*-Coumaric acid glucuronide

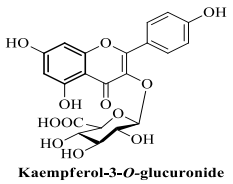

Apigenin

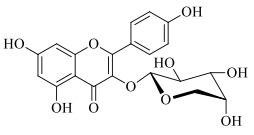

kaempferol

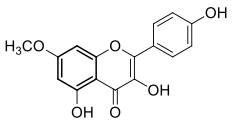

Quercetin

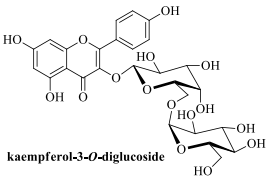

Myricetin


Luteolin


Kaempferol-3-*O*-glucuronide


kaempferol3-*O*-pentoside


Kaempferide (rhamnocitrin)


kaempferol-3-*O*-diglucoside

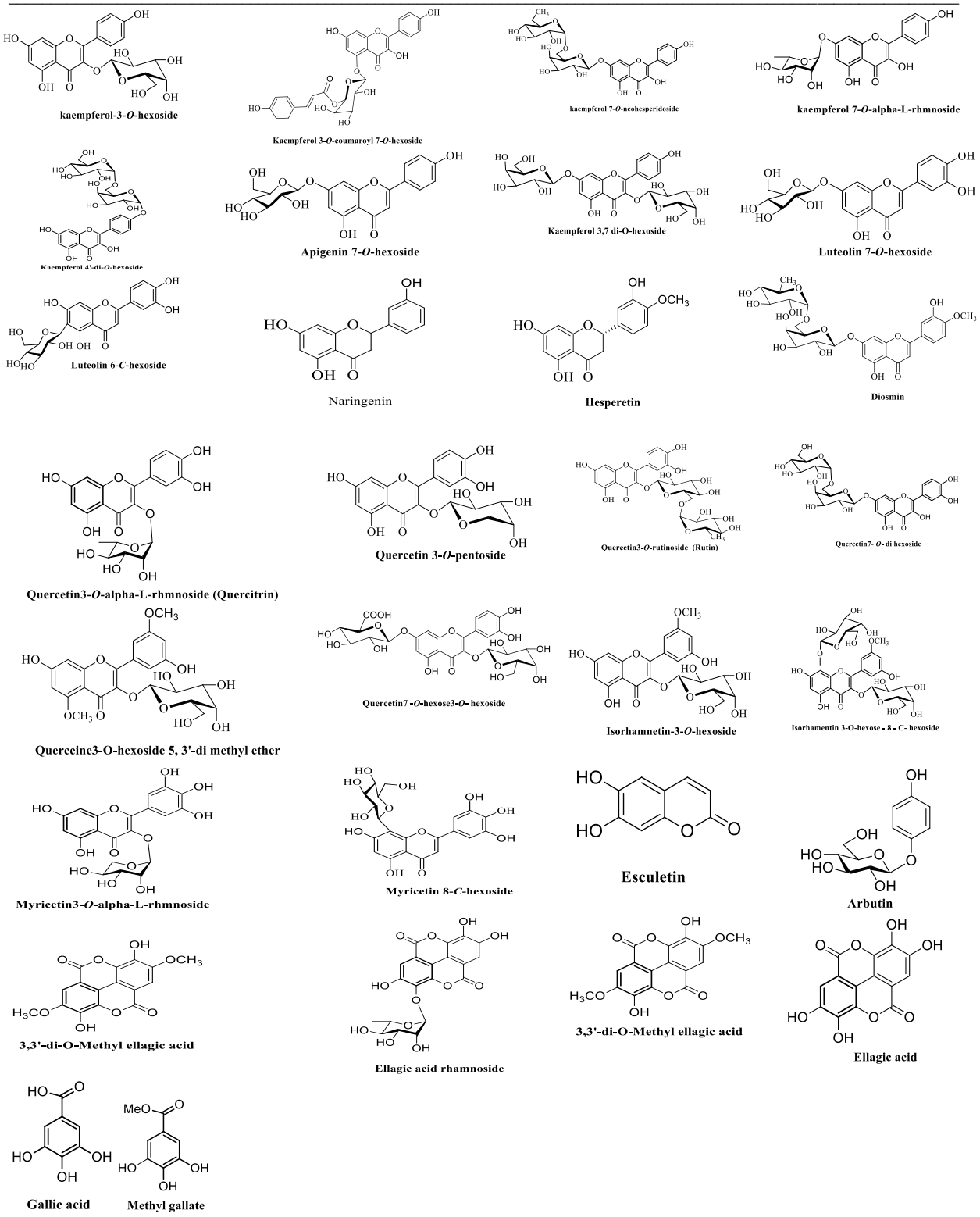



**Fig. 2. Structure skeleton of the main compounds tentatively identified from *C. indicum* aerial parts extract by HPLC/ESI-MS/MS**

**3.1.2.5. Tentative identification of flavones**

Compounds **41, 43 and 49** yielded the deprotonated aglycone fragment at m/z 268.98 indicating that they were derived from apigenine aglycone. These compounds were further confirmed from MS/MS spectrum .The characteristic ions at m/z 269.10[M-H-162]-, 268.98[M-H-(308)]-, and 268.89.10[M-H-$CH_3$]- suggested compounds **41, 43 and 49** as apigenine 7-*O*-hexoside, apigenine 7-*O*-rutinoside and apigenine 7-*O*-methoxide. Using the mass spectral information compounds **42**, **45** and **47** were detected as luteolin 6-*C*-hexoside, luteolin 7-*O*-hexoside and Chrysoeriol (3'-methoxy derivative of luteolin) **[19]**.

These compounds generated a main ion at m/z 285 and characteristic fragments of luteolin aglycone at m/z133.06, 151.03, 175.02. The appearance of two fragments of high abundance at m/z 285 [M-H-162]- and 284 [M-H-162]'- indicating the loss of a hexose unit. The 7-OH position was achieved by the predominant product at 285 [M-H-162]- coupled with the weak ion at m/z284 [M-H-162]'- **[13]**.

**3.1.3. Tentative identification of tannins**

Ellagi and gallo tannins were tentatively established in *C.* indicum by rapid separation using HPLC-ESI-MS-MS. Based on retention time; molecular formulae and accurate mass measurements.Four isomers of tannins were detected and tentatively confirmed as in **Table1**.

**3.1.4. Tentative identification of coumarin**

Only two coumarins (**53&54**) were tentatively established as Daphnetin (7,8-dihydroxycoumarin) and Esculetin (6,7-dihydroxycoumarin), respectively.

Only two coumarins (**53&54**) were tentatively established as Daphnetin (7,8-dihydroxycoumarin) and Esculetin (6,7-dihydroxycoumarin), respectively.

**3.2. Assessment of antioxidant activity using different assays**

The AME of CIA already owns antioxidant property show through the scavenging of DPPH radical. The percentage of DPPH radical scavenging inhibition was gradually increased with increasing the concentration. The highest investigated concentration of the total extracts (25µg/ml) recorded a promising radical scavenging activity (90.65±0.33 %) typically as the standard antioxidant BHA (90.44±0.29%).The lowest DPPH radical scavenging activity found to be 29.83±0.20 % at the concentration 5µg/ml **(Figure 7A)**.

Concerning to ABTS radical scavenging activity results in **Figure 7B**revealed that all the investigated concentrations of CIA extract ABTS radical ability even at a low concentration (5µg/ml) which found to be 19.65±0.37% .The Radical scavenging activity on ABTS increased gradually by increasing the concentration of the extract to reach 91.39±0.33% at the concentration 25µg/ml which is higher than that recorded by the standard antioxidant BHA (87.48±0.32%) at the same concentration. Furthermore the following concentration (20µg/ml) possesses ABTS radical scavenging activity (81.48±0.52) which considered as near as to the standard BHA.

Reducing power capacity assay measures the reducing potential of an antioxidant reaction evaluating Fe3+/ Fe2+conversion. The reduction capacity is correlated by the presence of chemical structure that exerts the action by breaking the free radical chain by donating a hydrogen atom **[20]**. The illustrated results in **Figure 7C**clearly showed that the reducing power capacity of CIA is high at low concentrations .The higher concentrations 20 and 25 µg/ml exhibited reducing power ability 1.12±0.016 and 1.34±0.027 respectively, which found to be higher than that obtained by the standard antioxidant BHA (0.95±0.011). Similar results were recorded byBhuiya N M **[21]**.The reported resulted showed that the reducing power activity of the CIA extract is higher than the standard in every concentration. It indicates a strong antioxidant capacity of the plant extract in a dose-dependent manner. They also concluded that the presence of flavonoids, including the flavonoids glycoside or any other polyphenols, could play a key role in the observed antioxidant activity.

FRPA of CIA extract **(Figure 7D)**takes the manner of the previous antioxidant assays, thus increasing the concentration of the extract concretely increase the ferric reducing power ability to reach 1943±30.20 µmolTrolox /100g at the concentration 25 µg/ml and decreased to 564±15 µmolTrolox /100g at the concentration 5µg/ml. Similar results were obtained by Bantho et al., 2023 they reported that FRAP results of *C. erythrophyllum* leaf and stem bark extracts varied from 6% to 97% inhibition depending on the extract concentration and the extraction solvent**[22]** . The results obtained from the divergence assay directly correlated with those obtained from the DPPH assay, indicating that the antioxidant activity is concentration dependent.

Metal chelation capacity of CIA extract **(Figure 7E)**was found to be slight lower than that obtained using DPPH, ABTS radical scavenging activity, reducing power and ferric reducing power assays. The highest metal chelating activity recorded with the concentration 25 µg/ml found to be 53.49±0.38% followed by the concentration 20 µg/ml (39.19±0.25%) while the metal chelating activity of the synthetic antioxidant BHA found to be 85.36±0.22 at the concentration 25µg/ml). These results may be due to the presence of specific bioactive secondary metabolites in CIA extract such as Phenolic acids, flavonoids especially (quercetin and kaempferol derivatives). Slight differences in

results between individual assays may be due to the nature of the chemical constituents and the mechanism of action of each antioxidant assay [23, 24].

Plants have been considered as a rich source of dietary antioxidants. Antioxidant activity was determined using different mechanisms. Polyphenols include flavonoids, tocopherols, tannins, glycosides and other organic acids. These compounds were found to exert a synergistic antioxidant effect. Presence of polyphenols may act as a key role in antioxidant activity observed [25]. Phenolic compounds can be considered the most abundant plant metabolites and well known for their antimutagenic, anti-inflammatory, apoptosis-inducing, antioxidant, and anti-carcinogenic effects, which primarily due to their phytoalexin potential [26].

Flavonoids are the largest group of plant metabolites within the class of phenolic compounds. Flavonoids also known for their anti-cancer, anti-viral, anti-allergic, anti-inflammatory, cholesterol-lowering, antioxidant and antimicrobial properties. Fernández et al. and Abd El-Rahman et al. determined the antioxidant activity of *Quisqualis Indica* extract using DPPH radical scavenging activity and stated that scavenging concentrations (SC50) were ranged from 24.38 to 72.10µg/ml ,while SC50 of ascorbic acid as standard found to be7.45µg/ml [27, 28]. They also investigated that reducing power capability results (abs.at 700 nm) are found to be in the following order: n-BuOH 0.680> Defatted 90% MeOH (0.465)> EtOAc (0.405)> 90% MeOH (0.225)>$H_2O$(0.90), compared with ascorbic acid as a standard (0.985) at a concentration of 200 µg/ mL. Their results are similar to our obtained results using lower concentrations (5-25 µg/ml). In a parallel direction similar results investigated by Barku et al., 2016, which confirmed that the DPPH radical scavenging ability, ferric reducing antioxidant power (FRAP) and total antioxidant capacity(TAC) of *C. dolichopetalum* for various solvent extracts increase with increasing extract concentration[29]. They suggested that the antioxidant activity of the extract may be attributed to that the high content of the polyphenolic compounds of the extract which donates hydrogen atoms to scavenge radicals, or causes electron transfer and reduces catalyzing metal ions oxidation process. Antioxidants work through enzymatic or non-enzymatic activity. They significantly impede oxidation of the oxidizable substrate when present at low concentrations [30]. Numerous biological and pharmacological effects of phytoconstituents, such as flavonoids and phenolic acids, which are frequently found in plants, have been documented. These effects include antioxidative, cytotoxic, anticancer, antibacterial, antiviral, and anti-inflammatory effects [31].

Protective impact of the antioxidants may be attributed to neutralizing of free radicals, which are harmful byproducts of normal cell metabolism. Antioxidants are created by the human body through a number of ways to combat oxidative stress. These processes include internal production of antioxidants as well as external supply from diet and/or supplements. Antioxidants serve as free radical scavengers by preventing and repairing ROS-related damage, which can strengthen the immune system and reduce the chance of developing cancer and degenerative disorders [32].

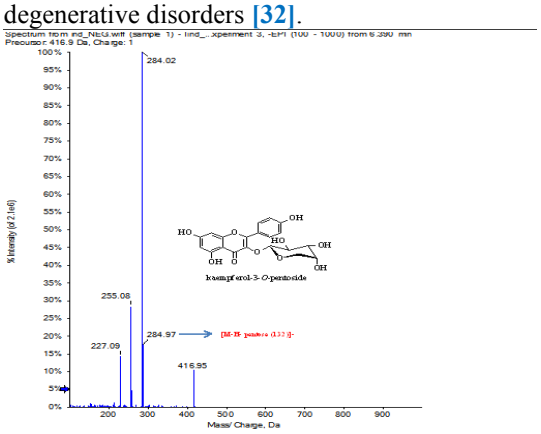



**Fig. 3. MS$^2$ spectrum of compound 20; the 3-OH glycosylation site.**

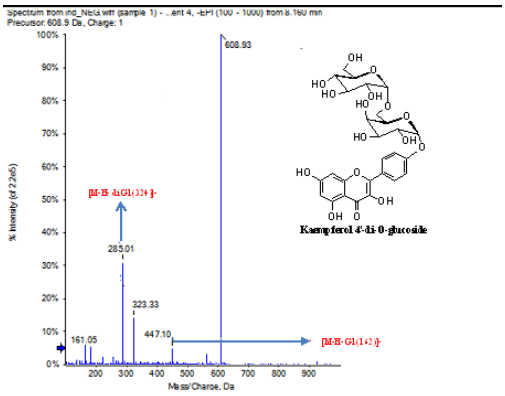



**Fig. 4. MS$^2$ spectrum of compound 28; the 4'-OH glycosylation site**

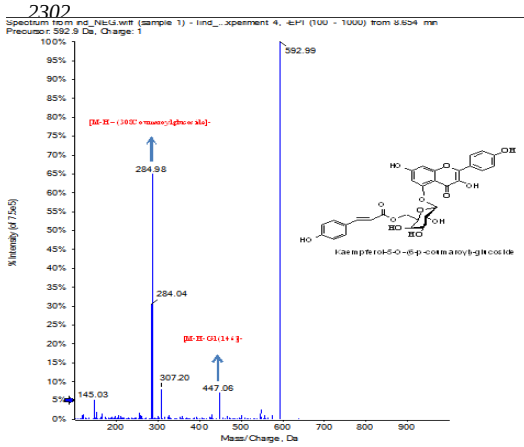



**Fig. 5. MS²spectrum of compound 34; the 5-OH glycosylation site**

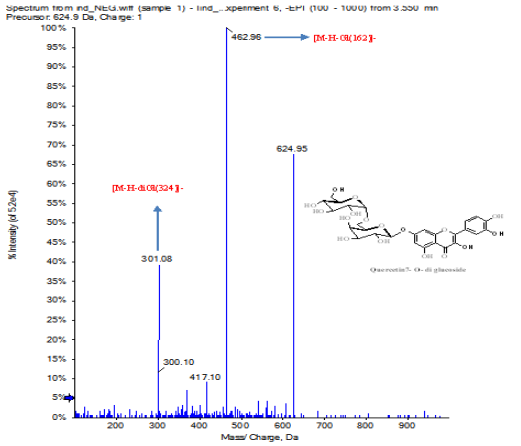
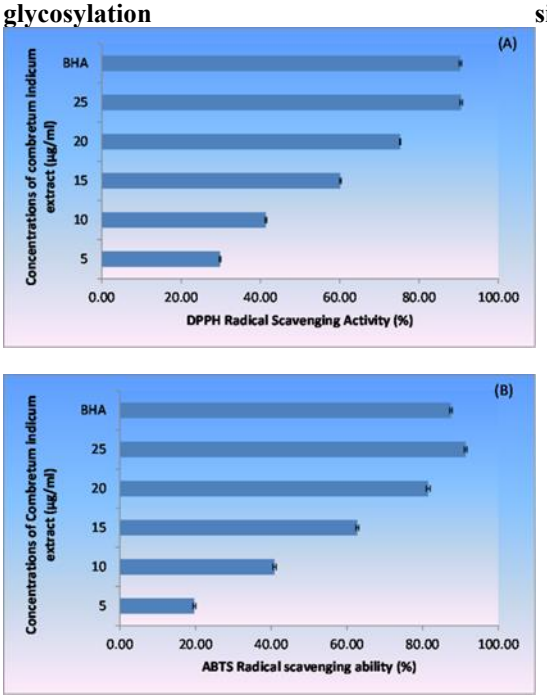



**Fig. 6. MS²spectrum of compound 14; the 7-OH glycosylation site.**



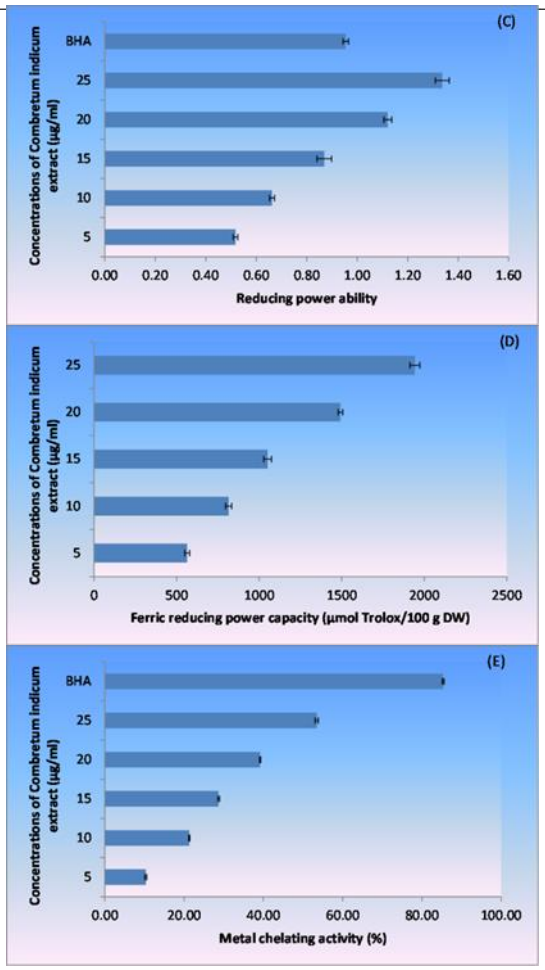




**Fig. 7. Antioxidant activity of different concentrations of *C. indicum* areal parts extract using different antioxidant assays: A) Scavenging ability on DPPH radical, B) Scavenging ability on ABTS radicals, C)Reducing power, D) FRAP Antioxidant capacity assay , E) Metal chelating ability . Values are averages and standard deviations for 3 independent experiments.**

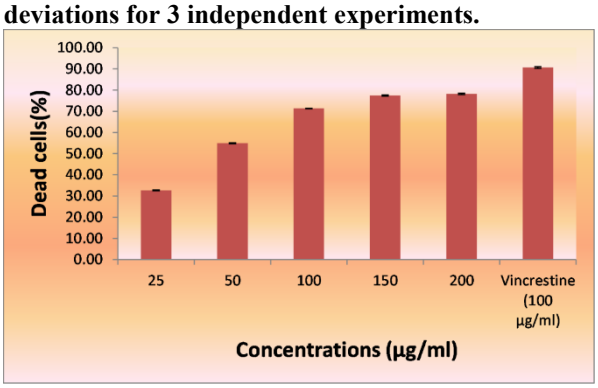



**Fig. 8. Effect of different concentrations of *C. indicum* aerial parts extract on the viability of EACC compared with Vincristine. Values are averages and standard deviations for 3 independent experiments.**

Quercetin and kaempferol'santioxidant activity was thought to play a cytoprotective effect against oxidative stress. According to some studies quercetin appears to shield cells from free radical damage through its antioxidant impact [33].

### 3.3. Assessment of *In vitro* antitumor activity using EACC

Herbal medicines have been proven to have therapeutic effects in the treatment and prevention of cancer. Cancer-related issues are lessened and the healing process is accelerated by herbal treatment [34]. Anti-cancer phytochemicals derived from plants may cause delaying in the transformation of normal cells into malignant tumours, induce neoplastic cell death, and inhibit angiogenesis and metastasis [35]. Figure 8 summarized the dead cell counting after incubation with different concentrations of CIA extract. By counting viable cells through trypan blue exclusions test, it was noticed that all the examined concentrations (25, 50,100,150 and 200 µg /tube) showed a noticeable effect on the viability of Ehrlisch ascites carcinoma cells. High concentrations (100,150 and 200 µg /ml) increased the percent of dead cells, which confirmed the tumoricidal effect of the extract with 71.35± 0.15, 77.40± 0.26 and 78.22± 0.30 % respectively, while the least concentration of CIA extract (25µg / tube) exhibited 32.72 % ±0.20 followed by 54.92 ± 0.26 % at the concentration (50 µg /tube).

Although, the standard drug Vincristine possess the highest antitumor activity (90.64± 0.39%) at the concentration 100 µg / ml, but the tumoral activity of the high concentrations of the investigated extract found to be as near as to the activity of the vincristine. Increasing the concentration of CIA extract didn't noticeably effects on the viability of the cells. The effect on the viability of Ehrlich ascites carcinoma cells may return to the presence of various natural polyphenolic components (e.g., chlorogenic acid, caffeic acid, isoquercitrin, and quercetin), in CIA extract, and consequently their active groups. The potentiality of these natural substances was subsequently confirmed through their potent role in the inflammatory processes and reactive species activity which are closely linked emergence and maintenance of various types of cancer. Similar results were recorded by Bantho et al., 2023; they studied the cytotoxic potential of the crude *C. erythrophyllum* leaf, stem and bark extracts (hexane, chloroform, and methanol) produced was evaluated against three cell lines.HEK293, HeLa and MFC-7) using the MTT assay [22].

In particular, very potent activity was observed with the methanolic stem bark extract Tested against HEK293 cells. Outgoing studies revealed that a methanolic stem bark extract emerged IC50 values less than 20 µg/mL make it a good choice for further development It is considered a conventional anticancer agent. Our results were reinforced by the results of Abd El-Rahman et al., 2016,which determined the cytotoxic activity of *Q. indica* fractions against hepatoma cell line (HepG-2),they reported that the IC50 values in µg/mL were organized as follows: $CH_2Cl_2$ (IC50 = 11.9), n-BuOH (IC50 = 17.9), defatted 90% MeOH (IC50 = 24.1), Pet. ether (IC50 = 35.1) and EtOAc (IC50 = 65.1) which confirmed the potent antitumor activity of *Q. indica* fractions .

It is well known that the anticarcinogenic effects of phenolic compounds are mainly due to its ability to induce cell cycle arrest and inhibit cell oncogenic signaling cascades stimulation of proliferation, angiogenesis, and apoptosis. Moreover, Phenolic compounds also modulate ROS levels and have pro- and anti-inflammatory effects [36]. In accord with some previous findings by Gibellini et al., 2010 suggested that quercetin promotes apoptotic cell death through its pro-oxidant activity and prevents the growth of tumors [33]. Therefore, the amount of quercetin in such types may influence their anticancer properties.In addition, the anticancer properties of CIA extract may be attributed to flavonoid molecules.Flavonoids have a wide range of anticancer actions, including modulating the activities of ROS-scavenging enzymes, participating in cell cycle arrest, inducing apoptosis and autophagy, and reducing the proliferation and invasiveness of cancer cells [37].

## Conclusion

The obtained results of the total extract of CIA clearly confirm this plant as a potential antioxidant and antitumor agents which enhances using it in the alternative medicines.This study contributed to promote the use of CIA in phytotherapeutics due to the biologically active compounds that were identified. The HPLC–ESI-MS/MS method presented here has been demonstrated to be an effective tool for the analysis of the components in a complex plant extract. In this investigation, HPLC-ESI-MS/MS method has been successfully established for rapid separation and identification of flavonoids and tannins in AME. Fifty nine phytoconstituents were tentatively identified; 10 phenolic acids, 29 flavonols including 11 quericetin derivatives, 6 isorhamnentin derivatives, 9 kaempferol derivatives and 3 myricetin derivatives , 9 flavones, 4 tannins, 2 flavanones and 2coumarins.

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**Conflicts of interest**

The authors declare that they have no competing interests that could influence the work reported in this paper.

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