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# BIOASSAY GUIDED ISOLATION AND IDENTIFICATION OF THE ANTIOXIDANT CONSTITUENT FROM *HOLOSTEMMA ADA-KODIEN SHCULT*

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## ABSTRACT

*Holostemma ada-kodien shcult* (Syn: *Holostemma annulare*) is a traditionally used in Indian herbal medicine. The objective of this study is to investigate the antioxidant activity of a bioassay-guided fractionation and its active components/compounds. Compounds were isolated by high performance thin layer chromatography (HPTLC) and preparative high performance liquid chromatography (pre-HPLC) and their structures were established by mass spectrometry (MS), Nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FT-IR) spectroscopic analyses. The antioxidant activity of ethyl acetate extract of *Holostemma ada-kodien shcult and* its fractions (CE, CF, EAF, MF and AF) were investigated using free radical scavenging activity, superoxide radical-scavenging assay, hydroxyl radical scavenging assay and assay of $FeCl_3$ power. The results revealed that the antioxidant activity of different fractionations such as CE and EAF showed prominent activity when compared with butylated hydroxyltoluene (BHT) in a dose-dependent manner. Two compounds were isolated from Fraction –D among them quercetin and acacetin were reported for the first time in *H. ada-kodien.* Quercetin showed a more prominent antioxidant activity potential at concentrations (10, 20, 30, 40 and 50µg/mL) when compared with the other compounds. In conclusion the ethyl acetate fraction proved to have significant therapeutic potential for antioxidant effect.

**KEYWORDS:** ***Antioxidant activity, Holostemma ada-kodien shcult, Butylated Hydroxyltoluene, Quercetin, Acacetin***

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# INTRODUCTION

*Holostemma ada Kodien shcult (H.ada-kodien shcult) (*Syn: *Holostemma annulare*) belongs to Asclepiadaceae family. It is also called as jivanti, arkapushpi, Kshira, dodi, suryavalli and widely distribute in tropical forest in India.[1] The plant is used as antidiabetic activity[2], antipyretic Activity[3], antibacterial activity[4], anti-inflammatory activity[3], antioxidant activity[4], other uses includes rejuvenative, aphrodisiac, expectorant, galactogogue, stimulant, and in ophthalmic disorders. *H. ada-kodien shcult* plant belongs to kingdom - plantae, phylum: magnoliophyta, class: Magnoliopsida, order: gentianales, family: asclepiadaceae, genus: holostemma and species: *Holostemma ada-kodien shcult*.[1] In this work, the study was to assess the evidence for the ethnobotanic properties of *H. ada-kodien shcult* using *in vitro* antioxidant activity experiments on the extracts as well as fractions and compounds obtained by bioassay-guided fractionation procedures. For this purpose free radical scavenging activity[6], superoxide radical-scavenging assay[7], hydroxyl radical scavenging activity[8] and assay of $FeCl_3$ [9] by using *in vitro* studies. The TPC (Total phenolic content) and TFC (total flavonoid content) of the methanolic extract was also determined by the folin-Ciocalteu method.[10] The identification of the phenolic compounds was performed by the $^1H$ & $^{13}C$ NMR, IR and mass spectroscopic methods, through interpretation and comparison of their spectra with those reported in the literature.

# MATERIALS AND METHODS

### *Plant materials*

The plant of *H. ada-kodien shcult* was collected in the month of November from the woods territory of the Tirumala hills, Tirupathi, Chittoor area. Andhra Pradesh (India). The plant material was taxonomically recognized by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateshwara University, Tirupathi, Andhra Pradesh, India and a specimen was kept in the herbarium. The plant was washed thoroughly to remove adhering soil and earthen matter, later on sliced to thin chips and dried in shade at room temperature and was ground to optimal coarse powder.

### *Chemicals and and reagents*

DPPH (2, 2-diphenyl-1-picrylhydrazyl), BHT (Butylated hydroxyltoluene) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DMSO (Dimethyl sulfoxide) were purchased from Triveni chemicals (Mumbai, India) NADH (Nicotinamide adenine dinucleotide-reduced), PMS (Phenazine methosulphate), EDTA (ethylene diaminetetraacetic acid), TCA (trichloroacetic acid), TBA and $FeCl_3$ was obtained from sisco research laboratories (Mumbai, India), the exception of Folin-Ciocalteu's reagent were purchased from sisco research laboratories (Mumbai, India).

### General experiment procedure

Infrared spectrum was obtained using bruker optics (Germany).$^1H$ NMR (400 MHz) and$^{13}C$ NMR (100 MHz) spectra were recorded on a Bruker Avance 400 spectrometer, with $CDCl_3$ as a solvent and tetramethylsilane (TMS) as internal standard. NMR experiments were performedin the same spectrometer. Thin layer chromatography (TLC) was performed on 0.20 mm precoated silica gel aluminum sheets (Merck Kieselgel 60F254). Spots were visualized with UV light (254 nm) and sprayed with vanillin sulphuric acid. Gravity columnchromatography (CC) was carried out by using Merck silica gel 60(70–230 mesh). HPTLC performed on 5X10 cm plates coated with 0.25mm layer of silica gel $60F_{254}$ ( Merck, Darmstadt, Germany). Samples were applied as 4mm wide bands and 6mm apart by using a Camag (Muttenz, Switzerland). HPLC Schimasze LC10 AD (Shimazu corporation, kyoto, japan)

### *Preparation of H. ada-kodien shcult extract, isolation and characterization of the active constituent*

The collected herbs of *H. ada-kodien shcult* were shade dried and powdered. Plant powder was then extracted three times at ambient temperature (50-60 °C), with 90% $CH_3OH$. During the extraction process the solvent was changed for every 24 h.[11] The $CH_3OH$ from the pooled extracts was then removed by distillation under reduced pressure at 50-60$^0$C for creating extracts of *H. ada-kodien shcult* (CE). The extracts were subjected to preliminary phytochemical investigation[12] and also for biological activity screening tests such as antioxidant activity. The 90% of $CH_3OH$ extracts were chosen for further isolation. The extracted solutions were suspended in distilled water and then sequentially extracted three times (2000mL × 3) with $CHCl_3$ (Chloroform) and EtOAc (Ethyl acetate), $CH_3OH$ (Methanol) and $H_2O$ extracts respectively.[13] All these fractions were screened for antioxidant activity. The potential active fractions were submitted on a silica gel column for further fractionation. These fractions were collected and combined after HPTLC analysis for yielding different fractions. The fractions possessing the potential pharmacological activity were consecutively re-chromatographed on a silica gel column. These fractions were further followed by semi-preparative HPLC using 55% methanol solution as mobile phase to obtain the final purified compounds. The purity of isolated compound was established by structural analysis and was confirmed by the interpretation of the spectral data (FT-IR, $^1H$, $^{13}C$ NMR and MS) and further tested for its pharmacological activities.

### *Determination of TPC*

An aliquot of 100 $\mu$L of extract was mixed with 2.5 mL of Folin-Ciocalteu phenol reagent and allowed to react for 5 min. Then, 2.5 mL of saturated $Na_2CO_3$ solution were added and allowed to stand for 1 h before the absorbance of the reaction mixture was read at $A_{725}$ nm.[14] The phenolic compound substance was resolved as phenolic compound content was determined as GAE (Gallic acid equivalents) using the linear equation based on the calibration curve $C = (c \times V/m)$ Where, C = total

content of phenolic compounds (mg/g plant extract in GAE); c = concentration of gallic acid obtained from calibration curve (mg/ml); V = the volume of the sample solution (mL); m = weight of the sample (g).[10] All tests were conducted in triplicate.

***Determination of TFC***

TFC of the plant crude extracts was determined spectrophtometrically using the Ghasemzadeh method[15] with little modification in the protocol. The test sample (1 mL) was placed in a 10-mL volumetric flask Mixed with Ethanol (60%, 8 mL) added after 6 min AlCl3 (10%, 0.2 mL), NaOH (4%, 0.6 mL) was added and then water to a volume of 10 mL. Read the absorbance of the reaction mixtures at $A_{430}$ nm (Spectrophotometer).A standard curve was plotted using quercetin as a standard 20-250 mg/mL. The TFC of the extracts was expressed as mg quercetin equivalents per gram of plant material on dry weight basis[16]: $H = (c \times V/m)$ Where, H = total content of flavonoid compounds (mg/g plant extract in GAE); c = concentration of quercetin obtained from calibration curve (mg/mL) V = the volume of the sample solution (mL); m = weight of the sample (g); All tests were conducted in triplicate.

***Free radical scavenging activity***

The DPPH radical scavenging activities of crude extract and fractions of *H. ada-kodien shcult* were tried by Brand-Williams [17]. Briefly, 0.2mL of the sample solutions of various concentrations was added to 1mL of 0.1mM of freshly prepared DPPH solution. The reaction mixture was shaken forcefully and absorbance at $A_{517}$ nm was determined after 20min at room temperature. Control sample was prepared contain the same volume without test compounds or reference antioxidants, while DMSO was used as blank. The reference antioxidant BHT was used as the positive control in all the assays. The radical scavenging activity was measured as a decrease in the absorbance of $DPPH^{\cdot}$ and calculated as follows

$$Scavenging\ effect\ (\%) = (A_{control} - A_{sample} / A_{control} \times 100)$$

Where $A$ $c_{ontrol}$ is the absorbance of the control and $A_{sample}$ is the absorbance of the extract or fractions or standard [9].

***Superoxide Radical-Scavenging Assay***

The superoxide radical-scavenging impact was determined by the method of Chun[7]. The reaction mixture with NBT, (1mM) in phosphate buffer (0.1 M, pH 7.4), NADH (1mM) with or without samples, and PMS (0.1mM) was incubated at room temperature for 10 min and the absorbance was reported at $A_{560}$ nm [8]. The inhibition percentage was calculated against a control the samples. The scavenging capacity was calculated using the equation as described for DPPH assay.

***Hydroxyl Radical Scavenging Assay***

The capacity of the extract and fractions to decrease hydroxyl radical-mediated peroxidation was carried out by the technique for Yu-ling.[6] Briefly, 0.5mL of 5.6mM 2-deoxy-D-ribose in $KH_2PO_4$−NaOH buffer (50 mM, pH 7.4), 0.2mL of 100 $\mu$M $FeCl_3$, and 104 Mm. EDTA (1 : 1 v/v) solution were added to 0.1mL of different concentrations of test samples, follow by 100 $\mu$L of 1.0mM $H_2O_2$ and 0.1mL of 1.0mM aqueous BHT. The reaction mixtures were shaken vigorously and incubated at $50^0$C for 45 min. Subsequently, 1mL of 2.8% TCA and 1mL of 1.0% TBA were added to each tube containing reaction mixture and samples were mixed well again and boiled in a water bath at 50 $^0$C for 45 min. The absorbance of solution was read at $A_{530}$ nm. The hydroxyl radical scavenging ability was calculated utilizing the equation as described for DPPH assay and the values are presented as means of triplicate analyses.

***Assay of $FeCl_3$ Power***

The FeCl3 assay was determined by the technique of Benzie and Strain with minor adjustments.[18] 1mL of extracts and fraction solutions (final concentration 10-50 µg/mL) was mixed with 2.5 mL of Phosphate buffer 0.2M, pH 6.6) and 2.5 mL of potassium ferricyanide (10 g/L), and then mixture was Incubated $50^0$C/ 20 min. then 2.5 mL of Tri chloro acetic acid (100 g/L) was added mixture, which was centrifuged at 3000rpm for 15 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 ml of $FeCl_3$ (1.0 g/L) and read the absorbance of the reaction mixtures at $A_{700}$ nm (UV-Visible Spectrophotometer). Increase absorbance of the reaction mixture indicates an increase in reducing power. The extracts, fractions and standard were calculated as follows.

$$Assay\ of\ FeCl_3\ power\ (\%) = (A_{test} / A_{control} - 1) \times 100$$

Where, $A_{test}$ is absorbance of test sample; $A_{control}$ is absorbance of test control; Control is BHT; Blank sol'n contained the same sol'n mixture without extraction and fractions

## STATISTICAL ANALYSIS [28]

Bartlett's test was performed on each set of data to ensure that variance of the sets are homogenous. In case of homogenous set of data ANOVA was performed to determine the treatment effects, and Dunnett's test was employed as appropriate by using Origin Pro 7.6 statistical software. In case of heterogeneous data, it was transformed using appropriate transformation. The variance was evaluated at 5% level of significance and the values were expressed as mean ± SEM and the $P<0.05$ was considered as statistically significant.

## RESULTS

***Preparation of H. ada-kodien shcult extract, isolation and characterization of the active constituent***

*H. ada-kodien shcult* plants collected herbs were shade dried and powdered. The plants powder (650 g) was extracted three times at ambient temperature (50-60 °C)

with 90 % methanol. During the extraction with solvents, the solvent was changed every 24 h. The methanol from the pooled extracts was removed by distillation under reduced pressure at 50-60 °C to create CE (62.5g). The extracts were subjected to preliminary phytochemical investigation and subjected for the biological activity screening tests. Therefore, the 90 % methanol extract was chosen for the following isolation. The extracted solutions (62.5 g) were suspended in distilled water, and then sequentially extracted three times (2000 mL × 3) with EtOAc, $CHCl_3$ and MeOH and $H_2O$ extracts, produce EAF(23.4 g), CF (6.2 g), MF (10.9 g) and AF (9.5 g) extracts, respectively. Among these fractions, the EAF was found to have the highest antioxidant activity. Therefore, the EAF (20 g) was submitted on a silica gel column (10 × 100 cm) using a gradient of ether-Et0Ac (5L) 100:0, 96:4, 92:8, 90:10, 80:20, and 70:30. Fractions of 5L were collected and combined after TLC analysis to yield 7 fractions of each plant (A – G). The fr-D possessed the potential antioxidant was consecutively re-chromatographed on a silica gel column (5 × 70 cm, 260 g) using a gradient of EtOAc (2L) 100:0,80:20,60:40,40:60,20:80. Fractions of 100 mL were collected and combined after HPTLC analysis to yield HAK-1 (2.45 g), HAK-2 (3.6 g). The extraction and isolation procedure of *H. ada-kodien shcult* is shown in Fig 1. HPTLC fingerprint the $R_f$ values of fraction HAK-1 (0.79) and HAK-2 (0.82) (Fig 2A, Fig 2B). These fractions were followed by semi-preparative HPLC using 55 % methanol solution as the mobile phase to obtain HAK-1 (36 mg) and HAK-2(49mg) of *H. ada-kodien shcult* (Fig 3), as a result, a known 3′,4′,3,5,7-Pentahydroxyflavone (quercetin) (HAK-1) (Fig 4A) and 5,7-Dihydroxy-4′-methoxyflavone (acacetin). (HAK-2) (Fig 4B) agrees in every respect (IR, NMR and MS) (Table 1).

***Preparation of H. ada-kodien shcult extract and isolation***

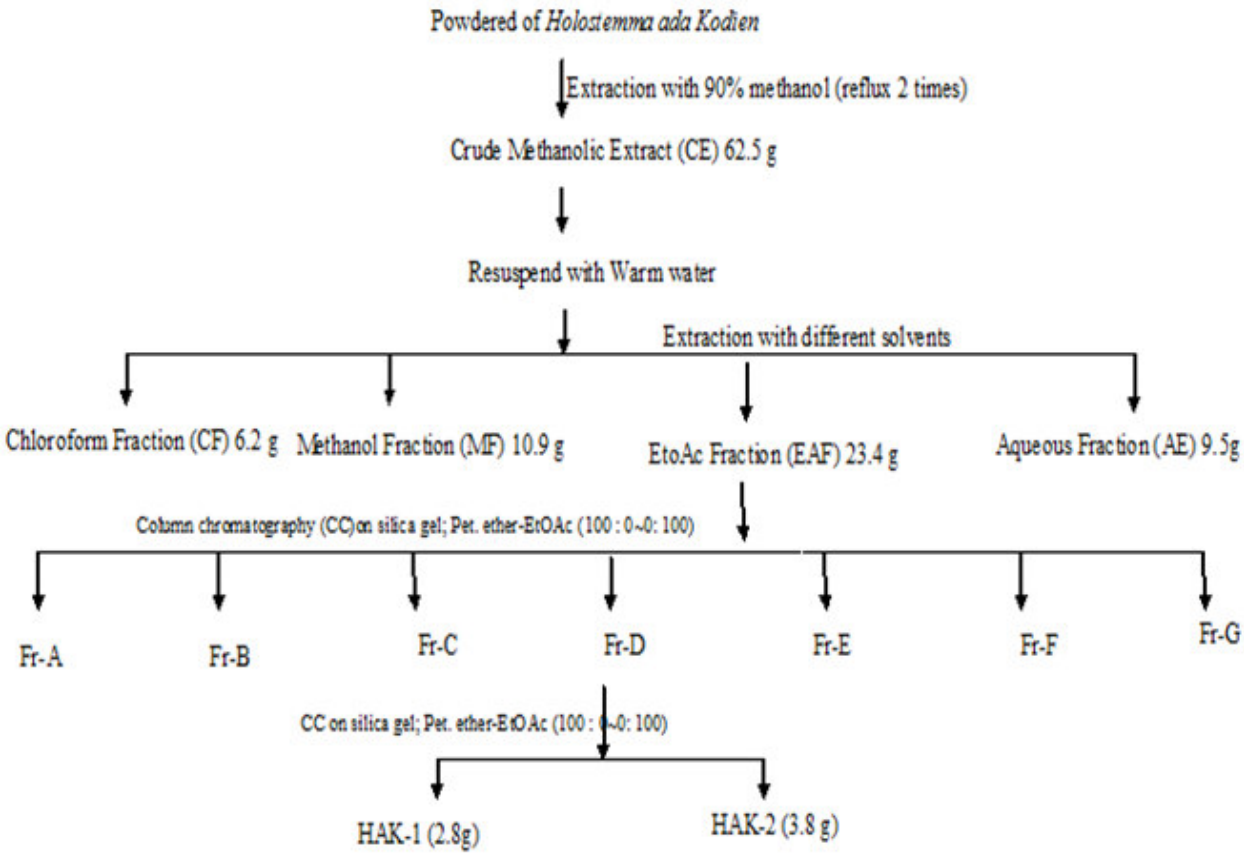
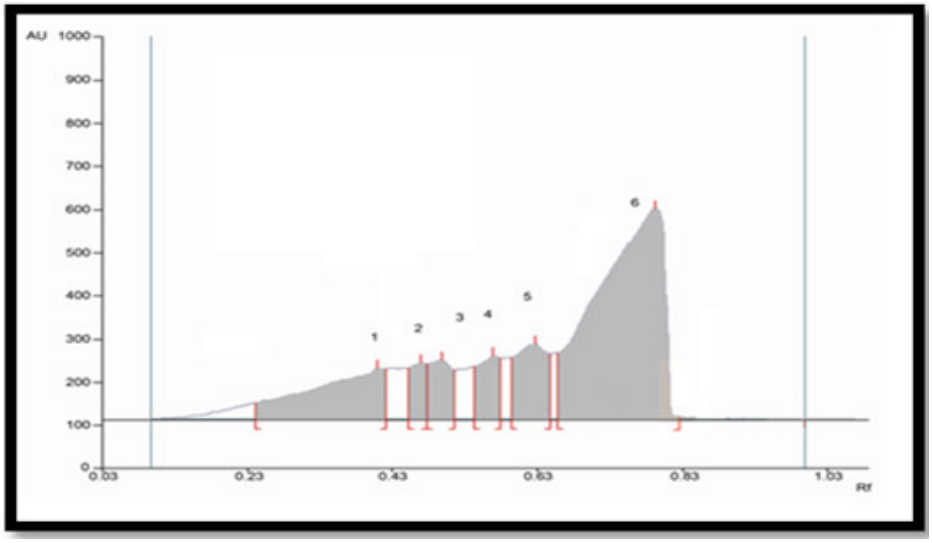
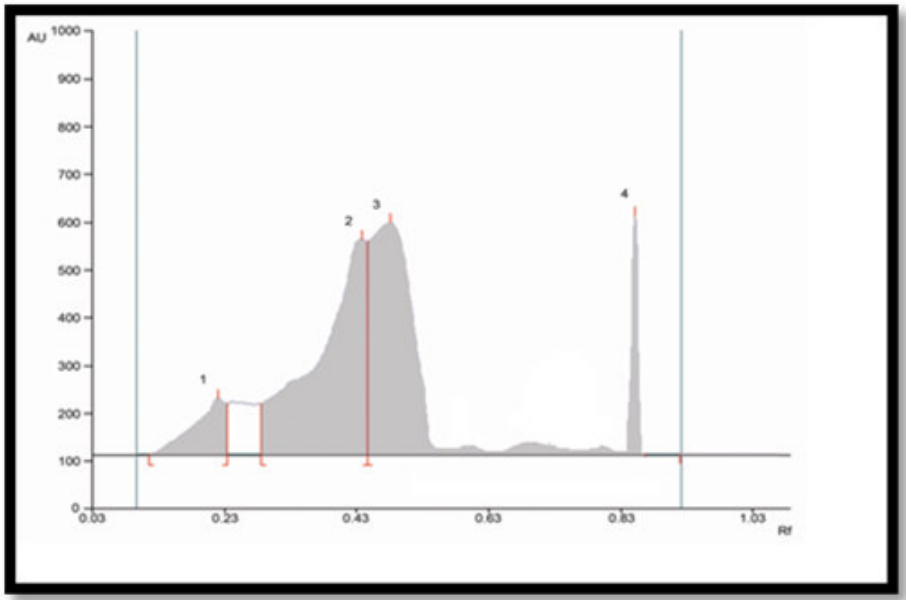



**Figure 1**
***Extraction scheme for the isolation of each compound from H. ada-kodien shcult***
***HPTLC fingerprints of H. ada-kodien shcult***




*(A)*




*(B)*

**Figure 2**
***HPTLC fingerprints of HAK-1 (A) and HAK-2 (B)***
**HPLC fingerprints of *H. ada-kodien shcult***

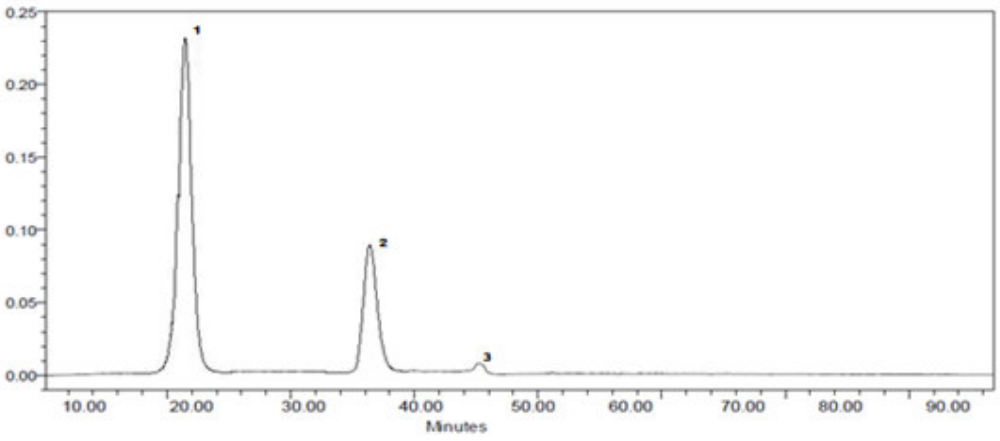



**Figure 3**
***HPLC fingerprints of fraction D (Fr.D) of EAF extract from H. ada-kodien shcult***
***Chemical Structures***

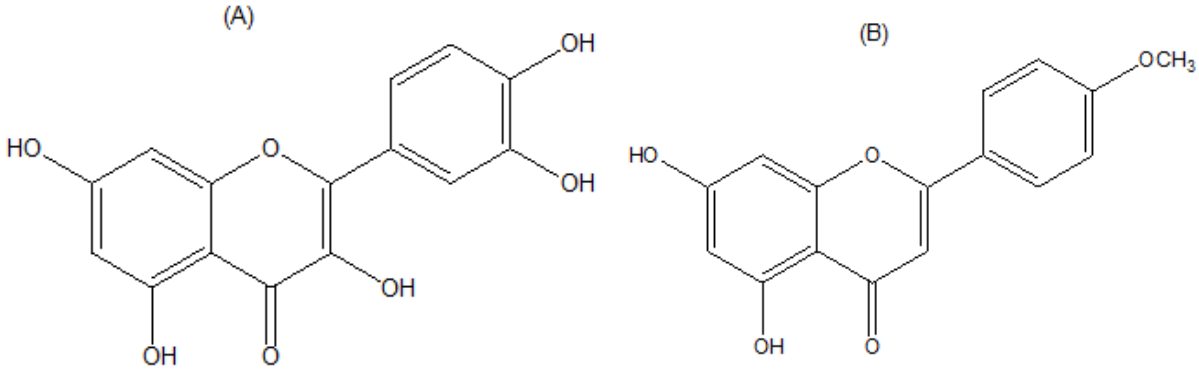



**Figure 4**
***Chemical structures of the two compounds HAK-1(A) and HAK-2 (B)***

**Table 1**
***The chemical constituents of HAK-1 and HAK-2 respectively, as elucidated by FT-IR,[1] H NMR, MASS.***

| Compound name | Solvent system | Rf Values at 254 nm | IR spectra | $^1$H NMR spectra (DMSO) | Mass spectrum |
|---|---|---|---|---|---|
| HAK-1 | Ethyl acetate: n-Butanol: n-Propanol: Methanol: water (60: 20:10:5:5) | 0.79 | OH-stretch-3422, C=O arylketone -1703, C=C stretch 1636; C-C Stretch 1508; C-O-C 1276; C-H stretch2921. | δ 7.9, 8.4 (2H, d,*j*8.4,1.9) , δ 4.9.6.8 (3H, s), δ 11.5,5.7,6.4 (5OH, s). | 302(M.F. $C_{15}H_{10}O_7$) m/z:M+303, 270,254,179. |
| HAK-2 | Ethyl acetate: Methanol: water (60: 30:10) | 0.82 | OH-stretch-3338 $cm^{-1}$, C-H stretch 2753 $cm^{-1}$,C=O aryl ketone 1630 $cm^{-1}$; C=C stretch 1618;C-C stretch-1530, 1434 $cm^{-1}$; C-CO-C -1181 $cm^{-1}$; C-O ether strech-1299 $cm^{-1}$, Ar-CH bending938,875, 830$cm^{-1}$, C-H stretch2917,2862 | δ 8.0, 7.6 (4H, d,*j* 8.8,1.6), δ 5.4, 5.5, 5.7 (3H, s), δ 8.4 (2OH,s), δ 3.94 (3H, s,-OCH3) | 285 (M.F. $C_{16}H_{12}O_5$) m/z:254,230,178. |

***Preliminary Phytochemical Studies***

The extract of *H. ada-kodien shcult* were subjected to preliminary phytochemical screening for the detection of various phytochemical constituents such as alkaloids, amino acids, carbohydrates, flavonoids, glycosides, mucilage, proteins, steroids, tannins and terpenoids.

***Determination of TPC and TFC***

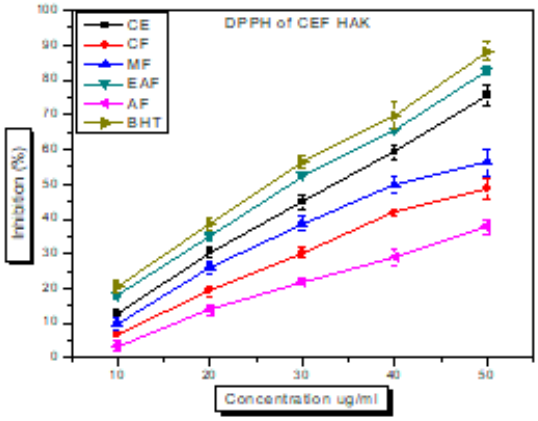
The total phenolic contains amount was calculated as quite high in CE was 24.68 ± 2.43 5 µg mg-1 GAE 100 $g^{-1}$ respectively. The total flavonoid contents in the examination of plant extracts are expressed in term of quercetin equivalent respectively. The values obtained for the con.c of TPC were measure in CE as 162.13 ± 3.29g quercetin 100g $^{-1}$. The CE may be responsible for its high free radical scavenging activity due to presence of hydroxyl groups in the polyphenolics and flavonoids. The key role of phenolic compounds as scavengers of free radicals is clearly emphasized. They were reported to eliminate radicals due to presence of hydroxyl groups and they contributed directly to antioxidant effect of system and it also played an important role in stabilizing lipid oxidation [29-30].

***Free radical scavenging Assay***

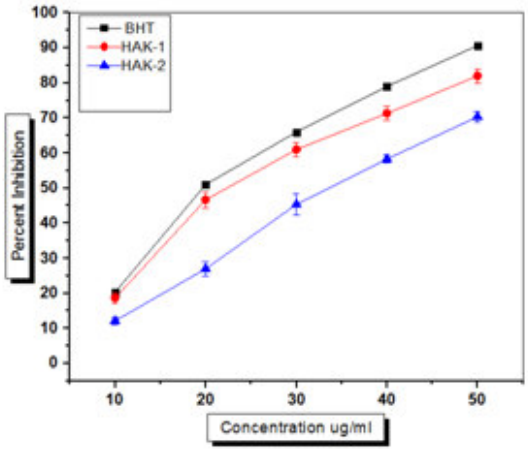
The substances are considered to be antioxidants, when they are capable of reducing the stable DPPH radical (purple) to the non-radical form DPPH-H (yellow) and thus they act as radical scavengers due to their hydrogen donating abilities.[19] The results of DPPH scavenging activity of all test samples are presented. (Gr-1) The scavenging activity of CE and fr-CF, MF, EAF, AF and BHT increased with an increase in sample concentration (10–50 $\mu$g $mL^{-1}$). The DPPH scavenging activity for CE and fr-CF, MF, EAF, AF and BHT was found to 75.59%, 48.77%, 56.36%, 82.82%, 37.63% and 88.17% respectively 50 $\mu$g $mL^{-1}$. The $IC_{50}$ values of CE and fr- MF, EAF and BHT was found to 33, 40, 27.5 and 26 $\mu$g $mL^{-1}$. It should be noted that the scavenging activity of EAF was found to be close to the BHT. The $IC_{50}$ values of scavenging activity on DPPH radical of extracts Fractions and BHT. From these obtained data, the CE and EAF were considered as an effective free-radical inhibitor as well as the primary antioxidants, which may limit free-radical damage that takes place in the body. The DPPH scavenging assay for isolated compounds HAK-1 and HAK-2 compared with BHT. The results were found to 82, 70.33 and 90.66% at 50 $\mu$g $mL^{-1}$. $IC_{50}$ values were 22, 33 and 20$\mu$g $mL^{-1}$ (Gr-2). It

should be noted that the scavenging activity of HAK-1 was found to be close to the BHT

*(Graph-1)*




*(Graph-2)*




*(BHT): Standard antioxidant; data represented as means ± SD ($n$ = 3)*

**Graph 1 and 2**

***DPPH radical scavenging activity of the crude extract and fractions of H. ada-kodien shcult (Gr 1); DPPH radical scavenging activity of the Isolated Compounds from H. ada-kodien shcult (Gr 2)***

***Superoxide Radical Scavenging activity***

The formation of ROS such as hydroxyl radical, $H_2O_2$, and singlet $O_2$ in living system was mainly due to the participation of superoxide anion radicals, either directly or widely through enzyme or metal catalyzed progression.[20] It was therefore anticipated to evaluate the relative interceptive capacity of the extracts and Fractions to scavenge the superoxide radical. From the data presented (Gr 3), it was noted that the CE and fr-CF, MF, EAF, AF and BHT showed the highest radical scavenging activities BHT and EAF (87.99% and 80.49%) at 50 $\mu$g mL$^{-1}$, $IC_{50}$ values was found BHT and EAF 24 and 29 $\mu$g mL$^{-1}$. The scavenging ability of compound on superoxide radicals was found to be moderate compared to EAF both plants. However, the scavenging activities of EAF were found to be very closer to that of BHT, which is considered to be a strong superoxide radical scavenger.The Superoxide Radical scavenging activity for isolated compounds HAK-1 and HAK-2 compared with BHT. The results were found highest radical scavenging activities HAK-1 and BHT (85.66 and 89 %) at 50 $\mu$g mL$^{-1}$(Gr 4), $IC_{50}$ values were 22 and 19$\mu$g mL$^{-1}$

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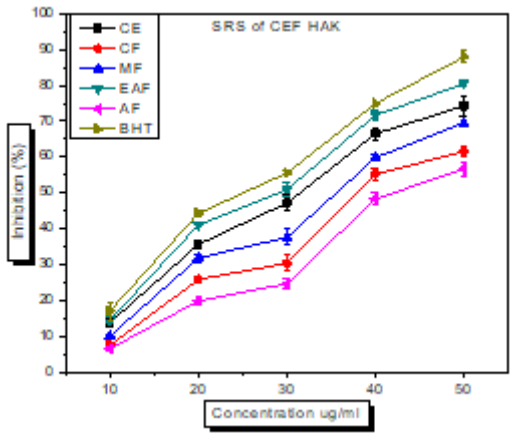
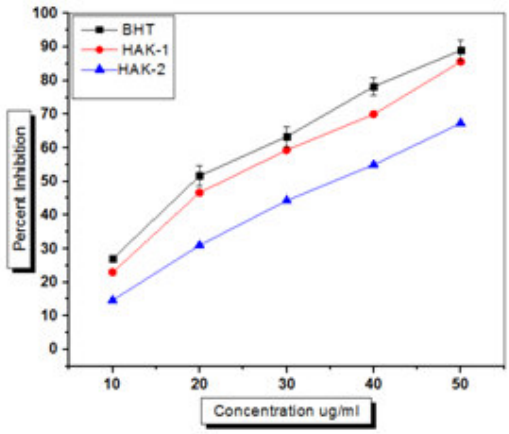
*(Graph-3)*




*(Graph-4)*




*(BHT): Standard antioxidant; data represented as means ± SD ($n$ = 3)*

**Graph 3 and 4**

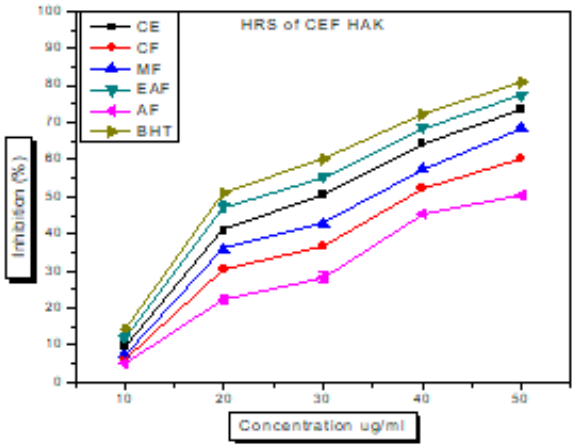
***Superoxide radical scavenging activity of the extract and fractions of H. ada-kodien shcult (Gr-3); Superoxide radical scavenging of the Isolated Compounds from H. ada-kodien shcult (Gr-4)***

***Hydroxyl Radical Scavenging Assay***

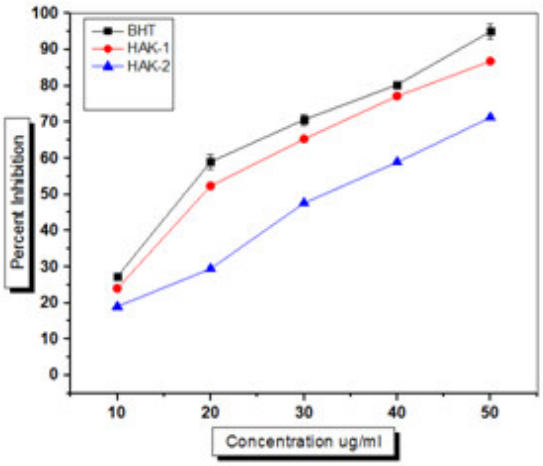
Hydroxyl radical, an extremely known reactive oxygen species, was competent to attack and spoil almost every molecule in the living cells.[20] They were also capable of stimulating lipid peroxidation process rapidly by attacking the fatty acid side chains of the membrane phospholipids. The scavenging activities of CE and fr-CF, MF, EAF, AF and BHT on hydroxyl radical inhibition are shown in (Gr 5). All the examined samples showed significant hydroxyl radical scavenging activity at 50$\mu$g

$mL^{-1}$ concentrations and the scavenging activity for CE and fr-CF, MF, EAF, AF and BHT 73.44%, 60.51%, 68.60%, 77.43% 50.04% and 80.69%. The $IC_{50}$ values of CE and fr-CF, MF, EAF, AF and BHT were found to 30, 38, 35, 22, 50 and 20$\mu$g $mL^{-1}$. This observed capacity of the extract and compounds to scavenge ·OH radical pointed out that the tested samples could considerably inhibit lipid peroxidation, since ·OH radicals are extremely distressed during peroxidation. The OH Radical scavenging activity for isolated compounds compared with BHT. The results were found highest radical scavenging activities HAK-1 and BHT (86.83 and 95%) at 50$\mu$g $mL^{-1}$, $IC_{50}$ values were 19 and 16.5$\mu$g $mL^{-1}$ (Gr 6).

***(Graph-5)***




***(Graph-6)***




***(BHT): Standard antioxidant; data represented as means ± SD ($n$ = 3)***

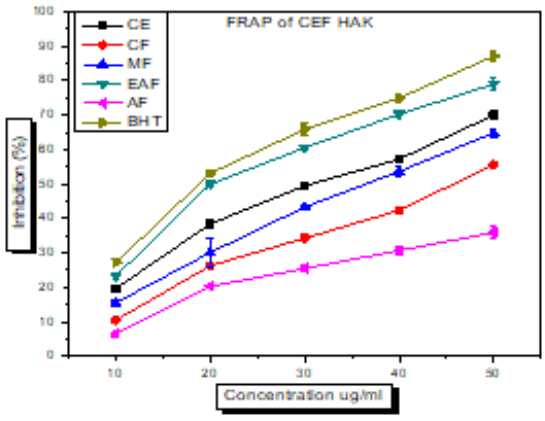
**Graph 5 and 6**

**Hydroxyl radical scavenging activity of the extract fractions of *H. ada-kodien shcult* (Gr-5); Hydroxyl radical scavenging activity of the Isolated Compounds from *H. ada-kodien shcult* (Gr-6);**

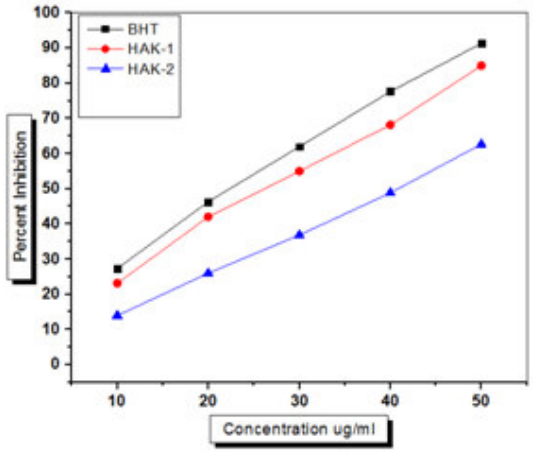
***Assay of $FeCl_3$ reducing power antioxidant component in dietary polyphenols.***[21]

The reducing properties are usually related to the presence of compounds which exert their action by breaking the free-radical chain by donating a hydrogen atom.[22] The results of reductive potential of plants extracts and fractions relative to BHT, a well-known antioxidant data, are shown in (fig-10). The reducing ability of CE- AF was in the range of 13.30-79.05% $\mu$MFe (II)/g. the $IC_{50}$ values of CE the fr-EAF was showing 20.50 $\mu$g $mL^{-1}$ and BHT 19 $\mu$g $mL^{-1}$ both. The FRAP values for the HAK were significantly higher than that of CE, CF, AF and MF, while the AF revealed the lowest FRAP values ( 6.57-35.95 $\mu$MFe (II)/g) at 50 $\mu$g $mL^{-1}$.The FRAP values for isolated compounds compared with BHT. The results were found highest radical scavenging activities HAK-1 and BHT (81, 85 and 91.33 %) at 50 $\mu$g $mL^{-1}$, $IC_{50}$ values were 30, 26 and 23 $\mu$g $mL^{-1}$(fig-11).The ferric reducing/antioxidant power (FRAP assay) is widely used in the assessment of the Graph 7 and 8.

***(Graph-7)***




***(Graph-8)***




***(BHT): Standard antioxidant; data represented as means ± SD ($n$ = 3)***

**Graph 7 and 8**

**FRAP scavenging activity of the extract and fractions of *H. ada-kodien shcult* (Gr-7); Fig11: FRAP scavenging activity of the Isolated Compounds from *H. ada-kodien shcult* (Gr-8);**

## DISCUSSION

Flavonoids, a major group of polyphenols, are considered to be the active principles in diverse medicinal plants and have been reported to possess numerous pharmacological properties. [23, 24] The most essential biological activityof flavonoids is mainly due to their antioxidant property by acting as radical scavengers[25], hydrogen donors, reducing agents[26], and peroxidation inhibitors.[27] Previous studies reported that the pharmacological effects of *H.ada-kodien shcult* such as antidiabetic activity [2]; antipyretic activity [3], antibacterial activity [4], anti-inflammatory Activity [3], antioxidant activity [5], were recognized due to the presence of the phenol and flavonoid compounds. In the present study, the isolated compound was identified as flavone and its antioxidant activities were examined by using various *in vitro* antioxidant models. Compound HAK-1 and HAK-2 yellow color solid, recorded data such as IR, $^1$HNMR and mass spectra conclusively proved that the isolated compound as 3′,4′, 3,5,7-Pentahydroxyflavone (Quercetin) and 5, 7-Dihydroxy-4′-methoxyflavone (Acacetin) were recognized due to the presence of the phenol and flavonoid compounds. In the present study, the isolated compound was identified as flavone compounds. Based on *in vitro* antioxidant results of the present work, the methanol extract and isolated compound from ethyl acetate fraction of *H.ada-kodien shcult* were believed to be an electron donor, capable of counteracting free radicals. The results of the present work also propose that the numerous pharmacological properties exhibited by *H.ada-kodien shcult* may be partly due to the presence of antioxidant flavone compound.

## CONCLUSION

The present study revealed the antioxidant and free-radical scavenging activities of extract *H.ada-kodien shcult* and its flavonoid, 3′, 4′, 5, 6-Pentahydroxyflavone and 5,7-Dihydroxy-4′-methoxyflavon and fractions by using in vitro antioxidant models. The ethyl acetate fraction exhibited highest free-radical scavenging activity, among the fractions. A bioassay-guided fractionation and purification of EAF resulted in the identification of the flavone compound, namely, 3′, 4′, 5, 6-Pentahydroxyflavone and 5,7-dihydroxy-4′-methoxyflavon. The measurement of antioxidant activity of the flavone compound, by using various in vitro antioxidant models, proved it to be a potent antioxidant compound. These results revealed that the ethyl acetate fractions and isolated compound exhibited interesting antioxidant properties and can be afforded as an essential basis for the use of *H.ada-kodien shcult* in the treatment of oxidative damages. Furthermore, these findings hold great perception in the development of alternative antioxidant agents, and still further work is warranted to sort out and characterize the active principles from other fractions, in order to establish their therapeutic efficacy and mechanism of action.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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