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ORIGINAL ARTICLE

Evaluation of Anti-Oxidant Property of *Acoras calamus* l. and *Commiphora Mukul* (Hook. Ex Stocks) Engl. for Medicinal Use

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ABSTRACT
Increasing burden of allopathic medicine on the society leads to anti-microbial resistance and cytotoxicity due to side effects. Therefore, in the present study we have evaluated the phytochemical screening and presence of phenolic and flavonoid compounds in methanolic extracts of rhizome of Acora scalamus and gum of Commiphora mukul for medicinal use. Rhizome of A. calamus and gum of C.mukul were extracted in methanol by maceration followed by their phytochemical analysis. Determination of total phenolic and flavonoid content was performed. ABTS and H_2O_2 antioxidant assays were deployed to evaluate total antioxidant capacity using ascorbic acid as reference standards. Analytical thin layer chromatography was performedto detect the presence of phenols and flavonoids. Total phenolic and flavonoid content of methanolic extract of A. calamus and C.mukul were 20.901±0.0693 and 5.692±0.0595, mg/g of gallic acid equivalent per gram of extract, and 13.37043±0.1072 and 24.55±0.263, mg/g of quercetin equivalent/gram of extract, respectively. ABTS and H_2O_2 % scavenging activity of A.calamus and C.mukul were found to be 99.52±0.225, 56.50±0.243,99.83±0.066 and 99.23±0.0884 respectively. A.calamus yielded three phenol bands (Rf =0.890,0.727 and 0.581) and two flavonoid bands (Rf = 0.951,0.548) whereas C.mukul yielded three phenol bands, (Rf = 0.726,0.579,0.290)and three flavonoid bands,(Rf= 0.967,0.822 and 0.5).The study shows that rhizome of A.calamusand gum of C.mukul possessed considerable amounts of phenol and flavonoid content and have high antioxidant activity in methanol.

Keywords: *Acoras calamus, Commiphora mukul,Phytochemical, Antioxidant, Thin layer Chromatography*

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INTRODUCTION

India is the largest producer of phytotherapeutics and recognized as the botanical garden of the world[1]. An antioxidant can be broadly defined as any substance that defers or inhibits free radical induced oxidative damage to a target molecule[2]. Phenolic compounds are stable radical intermediates, an important source of natural antioxidants which are known to be produced in large amounts by different plant parts such as seeds, bark of stems and roots and leaves in harsh environments with strong light intensities which mainly protects the plants against pathogens[3].Molecular structures of flavonoids have been known to be responsible for its strong antioxidant activity at very low concentration[4].*A. calamus* commonly known as "sweet flag" belongs to the family acoraceae and is a perennial and semiaquatic traditional indigenous medicinal plant distributed in areas of Jammu and Kashmir, Himachal Pradesh, Manipur, Tripura states, etc. in India[5].The roots and rhizome of *A.calamus* have been reported to exhibit various pharmacological activities including cardiovascular, anti-inflammatory, CNS etc.[6].*C.mukul*[syn.

Commiphorawigthii] (Family- Burseraceae) commonly known in India as "guggul" and in English as "Indian bdellium" is a therapeutically important ethnomedicinal plant since the ancient India Ayurveda system[8]. In India, it is abundantly found at arid regions of Gujarat (Kutch region),Rajasthan, Assam, Madhya Pradesh, and Karnataka[7, 8].Oleo -gum resin (pale yellow or brown coloured mass)secreted by *C.mukul* is a complex mixture of flavonoids, terpenoids, steroids, minerals and oils[9].Therefore, the present objectives of the study are to evaluate the phytochemical, total phenol content, total flavonoid content,in- vitro antioxidant activity and detect the presence phenolic and flavonoid compounds in methanolic extract of rhizomes of *A.calamus*and gum of *C.mukul*through thin layer chromatography.

MATERIALS AND METHODS

Collection and Extraction of Plant materials

A. calamus rhizome was collected from forests of Himachal Pradesh and *C. mukul* gum (Neeraj Traders, India) was purchased. Then, they were washed thoroughly with distilled water to remove the dust particles present in it, shade dried and grinded into a coarse powder. Maceration was carried out in methanol for 7 days with constant shaking in every 24 hours. After maceration, the mixture was filtered using Whatmann filter paper and the filtrate was evaporated using rotavapor at low temperature and pressure by a rotary evaporator to obtain the crude extract. All the extracts were preserved at 4^0C for further analysis[10].

Preliminary phytochemical screening Of Crude extracts

Methanolic extracts of *A.calamus* rhizome and *C.mukul* gum were subjected to different phytochemical tests for the detection of bioactive chemical constituents such as alkaloids, phenolics,tannins, flavonoid, carbohydrates, saponins and proteins using standard procedures[11. 12].

Determination of total phenols by Folin-Ciocalteu reagent method

Total phenolic content was determined by using Folin-Ciocalteu (FC) method through some modifications[13, 14]. FC reagent was freshly prepared (diluted 1:1 with de-ionized water) before use. Plant extracts (0.025mL) were mixed with FC reagent (2.5ml) and then allowed to stand for 5 min. at room temperature in order to allow complete reaction with the reagent. The reaction mixture was further neutralized with sodium carbonate solution (2ml, 7.5%, w/v). It was kept in dark at room temperature for 30 min. with intermittent shaking for blue color development. After 30 min, the absorbance was read at 765nm using UV-visible spectrophotometer (Thermo Spectronic) against a blank solution containing all the reagents except FC reagent which is replaced by an equal volume of distilled water. All the measurements were carried in triplicates and averaged. Methanolic solution of gallic acid served as standard (1mg/ml) phenolic compound (0.2, 1, 2, 3, 4, and 5µg/mL) for plotting calibration curve. Total phenol content in the extracts were expressed as Gallic acid equivalent (GAE) (mg of Gallic acid equivalent/g of dry weight sample) and was calculated by the formula[15]:

$$T = \frac{C*V}{M} \quad \text{[Eq. 1]}$$

Where, T=Total content of phenolic compound/g of plant extract, in GAE;
C=Concentration of Gallic acid established from the Calibration curve, microgram/ml;
V=Volume of methanolic extract; M=Weight of methanolic plant extract, g.

Determination of total flavonoid content by aluminium chloride colorimetric method

Total flavonoid content was determined by method described by Madaan et al. through some modifications[16].Plants extracts (0.5ml) were separately mixed with methanol (1.5ml), aluminium chloride (0.1ml,10%w/v), potassium acetate (0.1ml,1M) and distilled water (2.8ml). The reaction mixture was then incubated for 30 min at room temperature. After 30 min., using UV-visible spectrophotometer (Thermo Spectronic) the absorbance was measured at 415nm against a blank solution containing all the reagents except aluminium chloride which is replaced by an equal volume of distilled water. All the measurements were carried in triplicates and averaged. Methanolic solution of quercetin from 20 to 100 µg/mL served as standard (1mg/ml) flavonoid compound to obtain the calibration curve. The total flavonoid content was expressed as quercetin equivalent (QCE) (mg of quercetin equivalent/g of dry weight sample) and was calculated by the formula mentioned earlier for TPC[Eq.1]

Where, T=Total content of flavonoid compound/g of plant extract, in QE;
C=Concentration of quercetin established from the Calibration curve, microgram/ml;
V=Volume of methanolic extract; M=Weight of methanolic plant extract, g.

ABTS (2,2-azino-bis 3 ethyl benzothiazoline-6- sufonic acid) radical scavenging Assay

The radical scavenging activity of rhizome extract of *A.calamus* and gum extract of *C.mukul* were assessed by ABTS radical scavenging assay described by Re et al.[17] through some modifications. ABTS aqueous stock solution (7mM) and potassium persulfate stock solution (2.45mM) were freshly prepared. Equal

volumes of the stock solutions were mixed and allowed to react in the dark at room temperature for 12-16 h to obtain $ABTS^{\cdot+}$ free radical. After 12-16h, $ABTS^{\cdot+}$ working solution was diluted with methanol to obtain an absorbance of 0.70±0.02 at 734nm. Briefly, each methanolic extracts (10µl) were mixed with working solution of $ABTS^{\cdot+}$(2ml) and absorbance was taken 1 min. after initial mixing using UV-visible spectrophotometer (Thermo Spectronic). All the measurements were carried in triplicates and averaged. Ascorbic acid was used as standard. 1mg/ml stock solution of ascorbic acid was prepared and the calibration line between % inhibition and ascorbic acid was established using the following concentrations of ascorbic acid: 1,1.5,2,2.5,3,3.5,4,4.5, and 5 µg/ml. The total antioxidant capacity was calculated as percent inhibition of ABTS radical using the following equation,

$$\text{Percentage inhibition of ABTS}(\%) = \left(\frac{\text{Cabs}-\text{Sabs}}{\text{Cabs}}\right) * 100 \quad \text{[Eq.2]}$$

where Cabs is the control absorbance (ABTS radical + methanol); Sabs is the sample absorbance (ABTS radical + sample extract).

Hydrogen peroxide (H_2O_2) scavenging assay

H_2O_2 scavenging capacity of the extracts was evaluated using method described by Owaisi et al**[18]**through some modifications. Ascorbic acid was used as a positive control. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (0.05 mM, pH 7.4.Methanolic extracts (20 µl) and ascorbic acid (200 µl) in PBS was mixed with H_2O_2 solution (0.6mL,40 mM) to make a final volume of 2ml. The absorbance was read at 230nm UV-visible spectrophotometer (Thermo Spectronic) after 10 min. against subsequent blank solutions containing extracts (20µl) in PBS without H_2O_2. The amount of H_2O_2 radical scavenged by each extract was calculated as percent inhibition of H_2O_2 radical using the following equation,

$$\text{Percentage inhibition of H2O2}(\%) = \left(\frac{(\text{Cabs}-\text{Sabs})}{\text{Cabs}}\right) * 100 \quad \text{[Eq.3]}$$

where Cabs is the control absorbance (Ascorbic acid +H_2O_2 radical + PBS);
Sabs is the sample absorbance (H_2O_2 radical+ sample extract).

Thin Layer Chromatography for detection of phenols and flavonoids

Phenolic and flavonoid compounds in the methanolic crude extracts were separated by thin layer chromatography (TLC) using TLC plates coated with silica [19 – 21]. The plates were air dried and activated at 110° for 1 hr in *heat incubator* (Bioline Technologies, India). Samples (2mg/ml) were dissolved in methanol and concentrated in heat incubator for 10-15 min. Gallic acid and quercetin served as standard phenolic and flavonoid compounds (1mg/1ml). Ethyl acetate: formic acid: acetic acid: water (10:1.1:1.1:2.6, v/v/v/v) solvent system was used for separation of different phenolic compounds whereas for flavonoid separation methanol: chloroform: hexane: water (3:1:3:3) was used. Glass capillaries were used to spot samples and standard separately on the activated TLC plates up to 1.5cm from one edge of the TLC plate marked by pencil immediately after cooling at room temperature. The plates, with dried spotted samples, were gradually placed into the presaturated development tank, closed and left to develop. The mobile phase was allowed to travel until about 1 cm from the top end. After development, TLC plates were removed gently and solvent front was marked using a soft pencil line. For detection of phenols and flavonoids, TLC plates were air dried and then sprayed with a fine spray of 2% $FeCl_3$ solution and 1% ethanolic aluminium chloride solution, left to dry and then visualized under UV light at 254/365nm. The chromatograms were marked and retention factors were calculated using the formula:

$$\text{Rf value} = \frac{\text{distance travelled by solute}}{\text{distance travelled by solvent}} \quad \text{[Eq.4]}$$

RESULTS

Percentage yield of crude extracts

Approximately, 6-7g (70%) viscous mass was obtained from 10 g powdered rhizome and gum parts of *A.calamus*and *C.mukul* after maceration (Table 1A).

Preliminary phytochemical screening

Preliminary phytochemical studies revealed the presence of major bio-actives in rhizomes of *A.calamus* and gum of *C.mukul***(**Table 1B).

Total phenolic content

Total phenolic content was calculated using the following linear regression equation obtained from the standard plot y=0.4152x+0.446, r 2 =0.9906, where y is absorbance and x is the amount of gallic acid in µg(Fig 1A). The total phenolic content of the methanolic extract of *A.calamus* and *C.mukul* were 20.901±0.0693 and 5.692±0.0595, mg/g of gallic acid equivalent per gram of extract respectively (Fig 1B).

Total Flavonoid Content

Total flavonoid content was calculated using the following linear regression equation obtained from the standard plot y=0.0184x+0.118, r 2 =0.9944, where y is absorbance and x is the amount of quercetin in µg**(Fig 2A).** The total flavonoid content of *A.calamus* and *C.mukul* were 13.37043±0.1072 and 24.55±0.263, mg/g of quercetin equivalent/gram of extract (Fig 2B).

ABTS radical scavenging Assay

Radical scavenging activity of rhizome extracts of *A.calamus* and gum extract of *C.mukul* was also determined using ABTS method. ABTS scavenging activity (%) of standard ascorbic acid was calculated using the following linear regression equation obtained from the standard plot, y= 16.077x + 24.413, r^2 =0.981, where y is absorbance and x is the amount of quercetin in µg (Fig 3A). The ABTS % scavenging activity of *A.calamus* and *C.mukul* were found to be 99.52±0.225 and 56.50±0.243, respectively (Fig 3B).

Hydrogen Peroxide Radical Scavenging activity

Antioxidant activity in the two methanolic extracts were studied using the well-known H_2O_2 method and expressed in terms of %scavenging activity. The % scavenging activity of *A.calamus* and *C.mukul* were found to be 99.83±0.066 and 99.23±0.0884, respectively **(Fig 3C).**Total phenolics, flavonoid and antioxidant content of crude extracts of *A.calamus* and *C.mukul* are summarised in Table 2.

Thin layer chromatography for detection of phenolic and flavonoid compounds

Thin layer chromatography results showed the presence of different phenolic and flavonoid compounds in *A.calamus* and *C.mukul*(Fig 4) and (Table 3).

DISCUSSION

Preliminary phytochemical studies revealed the presence of bioactive constituents of our interest like phenol and flavonoids in methanolic rhizome extract of *A.calamus* and gum extract of *C.mukul*. In our study, the total phenolic content in *A.calamus* and *C.mukul* were found to be 20.901±0.0693 and 5.692±0.0595, mg/g of gallic acid equivalent per gram of extract, respectively**.** Previous studies reported that total phenolic content in methanolic rhizome extract of *A.calamus* ranged from 1.67±0.288 to10.42±0.180 mg/g gallic acid, whereas 12.45 mg GAE/g dw to 23.4 mg GAE/ 100 g dw phenolic content have also been reported[22, 23] which indicated that our result is quite consistent with results reported by other studies.The differences in the total phenolic content in same species of methanolic extract indicated that the total phenol content differed depending on the plant species, environmental factors, developmental stage and genetic make-up[24].In our study, the total flavonoid content of *A.calamus and C.mukul*were found to be 13.37043±0.1072 and 24.55±0.263, mg/g of quercetin equivalent/gram of extract, respectively. Total flavonoid content in methanolic extract of *A.calamus* rhizome reported by a previous study ranged from 0.50±0.053 to 24.50±0.322 mg/g catechin[23] whereas another study reported 54.2±0.15 mg quercetin /grams in ethyl acetate extract[5]. Our results hypothesized that extraction solvents might be a major factor influencing total flavonoid content amongst same species extracted by different solvents.

Our study showed that rhizomes of *A.calamus* and gum of *C.mukul* possessed high antioxidant activity. Previous studies reported guggulsterones as the chief bioactive compound present in *C.mukul* gum resin responsible for its *in vitro* antioxidant activity[25].Thin layer chromatography results revealed the presence of different phenolic and flavonoid compounds in *A.calamus* and *C.mukul*. The color and Rf value of *C.mukul* (CM3') matched with that of standard (quercetin) which indicated the presence of quercetin as the major flavonoid compound in *C.mukul* gum extract. This data might be supported by an earlier study which reported based on our quantitative flavonoid analysis and thin layer chromatography results; we can conclude that *C.mukul* gum resin has a high flavonoid content, "quercetin" being the chief flavonoid compound. Our findings are supported by a study which reported quercetin as the major flavonoid compound found in the flowers of *C.mukul*[26].

The present work revealed presence of bioactive compounds and the strong antioxidant activity of methanolic rhizome extract of *A.calamus* and gum extract of *C.mukul.* Both the plants showed the presence of phenols and flavonoids but *A.calamus* had a higher phenolic content whereas *C.mukul* showed a higher flavonoid content in methanol. Furthermore, on the basis of our results, it can be concluded that a pronounced free radical scavenging potential of *A.calamus* rhizome and *C.mukul*gum methanolic extract was observed which suggested a possible therapeutic application of *A.calamus*rhizom*e* and *C.mukul* gum as potential sources of natural antioxidants for development of bioactive compounds, which might help in prevention of oxidative stress. It has been reported that high phenolic content were associated with strong antioxidant activity[27]. Thus, we can conclude from our study that antioxidant potential of *A.calamus* and *C.mukul* might be possibly influenced by high phenolic and flavonoid compounds present in them. Since,*C.mukul* is IUCN listed, so proper usage of this phytotherapeutic drug should be done to

prevent depletion of this wonder drug. More research on *A.calamus* rhizome and *C.mukul gum* resin needs to be done to explore its immense phytotherapeutic potential.

Table1A: % yield of Methanol extracts from *A. calamus* and *C. mukkul*

Scientific Name	Family	Common names	Parts analysed	Medicinal uses	Yield % (w/w)
Acorus calamus	Acoraceae	Sweet flag, vaccha	Rhizome	Detoxifying agent, treatment of nervous disorders, reducing obesity, aromatic stimulant, febrifuge vermifuge and hypotensive	60%
Commiphora mukul	Bursuraceae	Guggul, Indian myrrh	Gum resin	Cardiac tonic, expectorant, skin complexion enhancer, carminative, hypercholesteremic and antibacterial	70%

Table 1B: Results of preliminary phytochemical screening of methanolic extracts of *A. calamus* and *C. mukkul* (+: present in low concentration, ++: present in moderate concentration, +++: present in higher concentration, -: absent)

Phytochemical	Name of Test	*A.calamus*	*C.mukul*
Alkaloid	Dragendorff's test	+++	+++
	Wagner's Test	++	++
Phenol	Ferric chloride test	++	+
Tannin	Ferric chloride test	+	-
Flavonoid	Alkaline reagent test	+	+++
Carbohydrate	Fehling's test	+++	+
	Benedict's test	-	-
	Barfoed's test	-	-
Saponin	Foam test	+	-
Protein	Xanthoproteic test	+++	+
	Ninhydrin test	-	-
	Biuret test	+	-

Table 2:Total phenolics, flavonoid and antioxidant content of crude extracts of *A.calamus* and *C. mukul*

Crude Extracts	*A.calamus*	*C.mukul*	*P*
TPC[a]	20.901±0.0693	5.692±0.0595	0.00001*
TFC[b]	13.37043±0.1072	24.55±0.263	0.00001*
ABTS[c]	99.52±0.225	56.50±0.243	0.00001*
H_2O_2[d]	99.83±0.066	99.23±0.0884	0.000361*

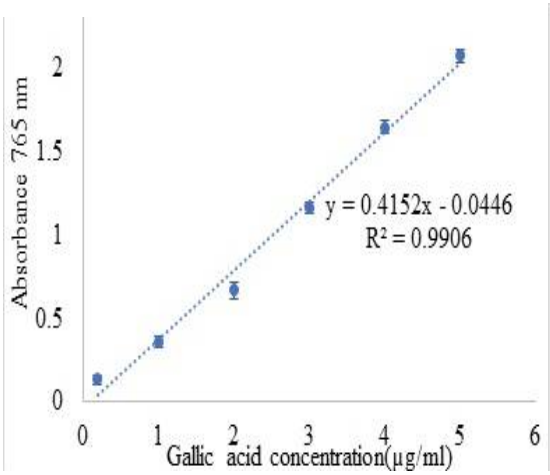
[a]Total phenolic content expressed as mg of gallic acid equivalent (GAE)/g of dry weight (dw).[b]Total flavonoid content expressed as mg of quercetin equivalent (QE)/g of dry weight (dw).[c]ABTS radical scavenging activity expressed as % inhibition.[d]H_2O_2 radical scavenging activity expressed as %inhibition. Values are mean±SD; *:$P<0.05$

Table 3A: Phenol analysis using 2% $Fecl_3$ solution

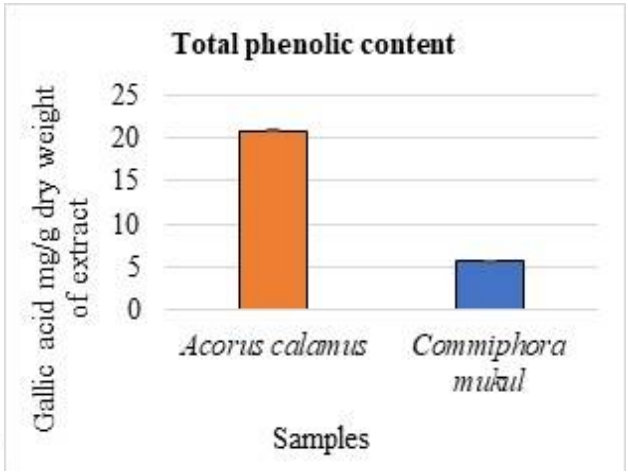
Crude extracts	No of spots	Rf value
Gallic Acid (Std)	1	0.909
A.calamus	3	0.890,0.727,0.581
C.mukul	3	0.726,0.579,0.290

Table 3B:Flavonoid analysis using 1% ethanolic aluminium chloride solution

Crude Extracts	No of spots	Rf value
Quercetin (Std)	1	0.967
A. calamus	2	0.951,0.548
C.mukul	3	0.967,0.822,0.5



1A



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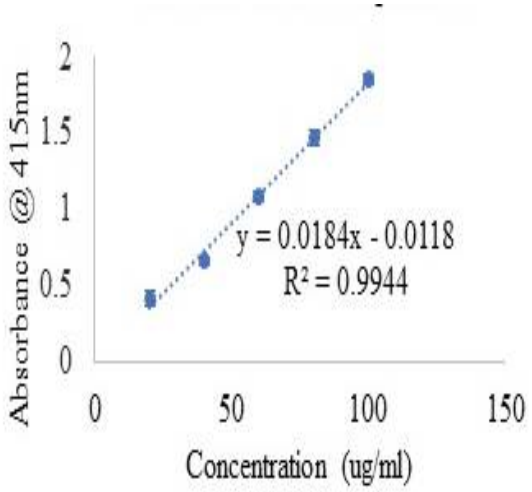

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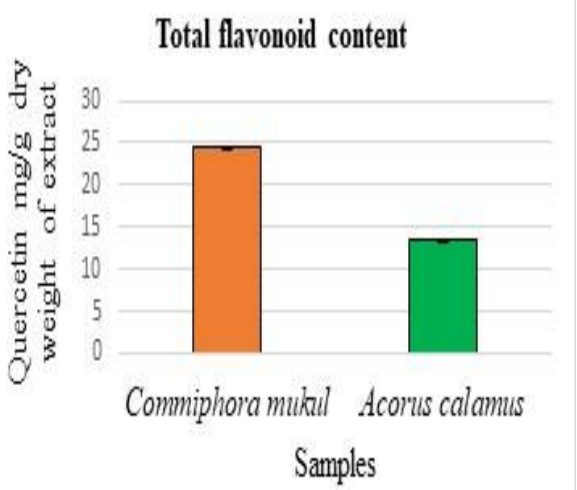
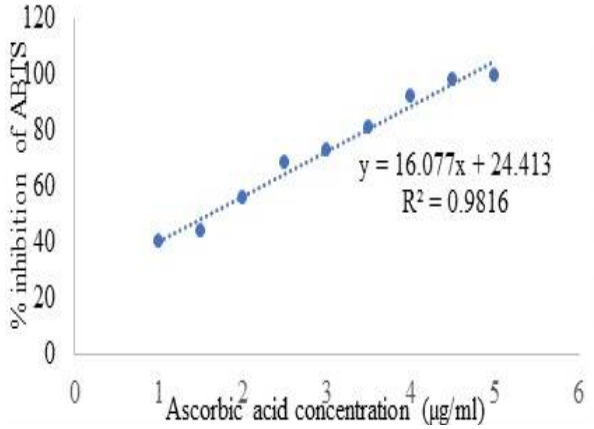


2B

Figure 1(A):Standard curve of Gallic acid; (B) Total phenolic content in crude extracts
Figure 2(A): Standard curve of Quercetin; (B) Total flavonoid content in crude extracts



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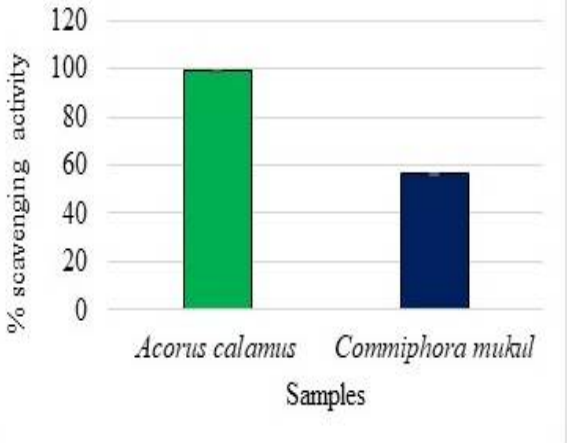

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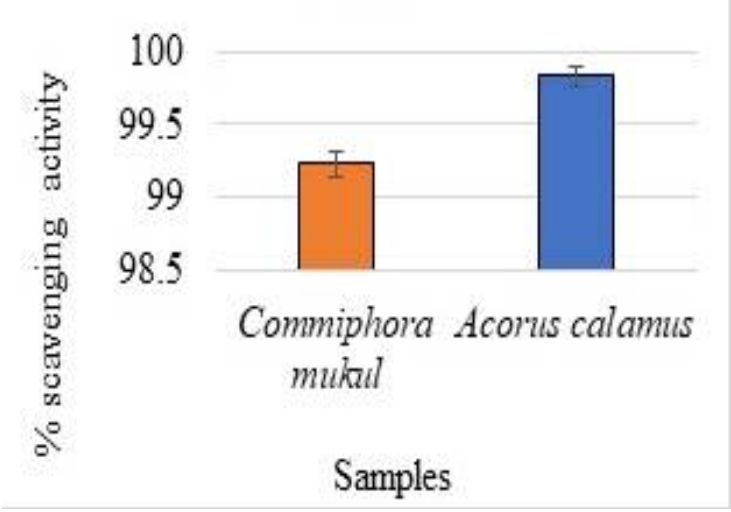

3C

Figure 3: (A) Standard curve of Ascorbic acid; (B) ABTS scavenging activity (%) of crude extracts; (C) Hydrogen peroxide scavenging activity (%) of crude extracts

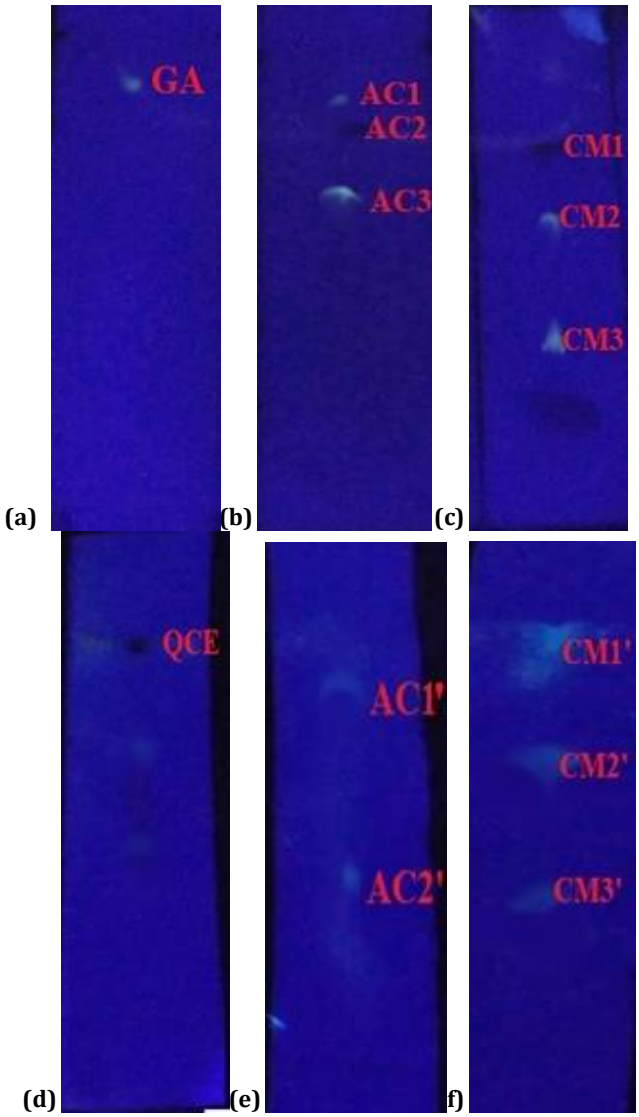

Figure 4. Chromatograms for phenols (a-c) and flavonoids(d-f) under UV:(a)Gallic acid standard (b)*A.calamus* (c)*C.mukul* (d)quercetin standard (e)*A.calamus*(f) *C.mukul*

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