

Full Length Research Paper

Antimicrobial efficacy of *Casuarina equisetifolia* extracts against some pathogenic microorganisms

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Casuarina equisetifolia (Casuarinaceae) is a plant that is used in folk medicine for the treatment of astringent, in diarrhea, cough, ulcers, toothache, lotion for swelling and diabetes. To evaluate the scientific basis for the use of the plant, the antimicrobial activities of leaves extract was investigated against 7 medically important bacterial strains, namely *Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Micrococcus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and 4 fungi. The antibacterial activity of aqueous and organic solvents was determined by agar well diffusion method. The most pronounced effect was shown by the methanol extract. The most susceptible bacteria were *S. aureus*, followed by *K. pneumoniae*, while the most resistant bacteria was *B. subtilis* followed by *Micrococcus*. The antifungal activity of aqueous and organic solvents was also determining. The most pronounced effect was shown by ethanolic. The most susceptible fungi were *Aspergillus flavus* while the most resistant fungi were *Candida albicans*. The high performance liquid chromatography (HPLC) analysis indicated the presence of 8 phenolic compounds as major active constituent (gallic, protocaticic, chlorogenic, syringic, p.hydroxy benzoic, p.coumaric, vanillic and salicylic acid). Result obtained indicated variable difference in concentration of the compounds in *C. equisetifolia* leaves extract. Results of HPLC showed gallic, salicylic and protocaticic in high concentration of 19.18, 11.57 and 6.84 µg/g, respectively. The concentration of other phenolic compounds ranged from 1.63 to 4.70 µg/g. The least concentration was chlorogenic with 1.63 µg/g.

Key words: *Casuarina equisetifolia*, antibacterial, antifungal, phytochemical constituents, crude extract.

INTRODUCTION

Casuarina equisetifolia (Casuarinaceae) is an handsome tree with drooping branches, 10 to 50 m high (Mhaskar et al., 2000); it is found in dry hill sides and open forests of India, Srilanka and to Australia. The following phytoconstituents have been isolated from the plant so far: kaempferol, quercetin (El-Ansari et al., 1977), alicyclic acids (shikimic and quinic acids), amino acids (Madhusudanamma et al., 1978), taraxerol, lupenone, lupeol, gallic acid, sitosterol (Rastogi and Mehrotra, 1998), catechin and gallo catechin (Roux, 1957; Madhulata et al., 1985). Casuarinaceae family were important as food source to the Aboriginal people of Australia prior to European settlement (Nash, 2004).

Casuarinaceae family also had a role as traditional bush medicines.

The bark of *C. equisetifolia* was used as astringent (Mhaskar et al., 2000) and to treat diarrhea, dysentery, headache and fever (Maiden, 1989) cough, ulcers, toothache, lotion for swelling (Wealth of India, 1992) and diabetes (Prajapati, 2003). Pharmacological investigations of bark and wood showed significant anticancer and anthelmintic activities (Aher et al., 2006, 2008). The biological activates, viz. anticancer, antibacterial (Wealth of India, 1992; Parekh et al., 2005; Cock, 2008) and hypoglycemic, antifungal (Han, 1998) of the leave has been reported. The present study was designed to evaluate the phytochemical and antimicrobial properties of *C. equisetifolia* leaves extracts, material obtained from Jeddah city, Saudi Arabia.

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MATERIALS AND METHODS

Collection and preparation of samples

Samples of *C. equisetifolia* leaves were collected during May, 2011 from Albahah city (19°98' 28"N; 41°52' 50"E) southwest Saudi Arabia from cool slopes at 2050 m.a.s.l. Species status of this plant was fervid at Faculty of Sciences Herbarium (Serial No. 1596), King Abdulaziz University, Jeddah. The plant leaves were brought to the laboratory, washed in running tap water to remove debris and dust particles and then rinsed in distilled water for 5 min.

Preparation of *Casuarina equisetifolia* extracts

Ten grams (10 g) of dried *C. equisetifolia* leaves were thoroughly washed in running water prior to cutting into small pieces by blender 1 to 2 mm. Extraction was done by adding 100 ml of distilled water and organic solvent (ethanol, petroleum ether, diethyl ether, ethyl acetate, chloroform and methanol extract) (1:10 w/v). Under cold conditions for 48 h, the solvent extract was filtered through a filter paper. The extracts solutions were evaporated under reduced pressure at 40°C until dryness; subsequently, the extract was diluted by dimethyl sulfoxide (DMSO) and stored in 20°C until analysis according to Boeru and Derevici (1978)

Phytochemical screening

Identification of phenolic acids

Vanillic, ferulic, syringic, p-coumaric, p-hydroxybenzoic and protocatechuic were subsequently checked for purity by high pressure liquid chromatography (HPLC). HPLC grade water and MeOH were used for all analyses. Phosphoric acid buffer was made using HPLC grade $\text{NH}_4\text{H}_2\text{PO}_4$ and H_3PO_4

Phenolic extraction and hydrolysis

Phenolic compounds in plant were extracted as described by Mattila et al. (2005) with some modifications. Approximately, 15 ml of 4N NaOH was added to 200 ml of each concentration of water extract in 50 ml Pyrex centrifuge tube purged with nitrogen and shaken for 2 h in dark with a wrist - action shaker. After phenolic acids were liberated by alkaline hydrolysis, samples were acidified with ice - cold 6 N HCl to reduce pH to between 1 and 2. Samples were centrifuged at 3000 g and the supernatant was decanted into a 250 ml separator/funnel. The supernatant was extracted with ethyl acetate (3 × 50 ml) with shaking for 10 s and the mixture was allowed to settle for 5 min between extractions. Ethyl acetate fractions were collected and pooled. The remaining pellet was diluted with 15 ml of distilled H_2O , vortex distributed and re-centrifuged at 3000 g. The second supernatant was re-extracted with ethyl acetate (30 × 50 ml) as before and all ethyl acetate fractions were pooled. The phenolic acids-rich ethyl acetate fraction was dried by addition of anhydrous sodium sulfate and concentrated using a rotary vacuum evaporator at 35°C to dryness. The phenolic acids-rich residue was re-solubilized in 2.5 ml of MeOH and stored in a dark prior to separation and quantification by HPLC within 24 h of extraction.

HPLC analysis

Phenolic acids were separated by Shimaduz (Kyoto, Japan) HPLC

apparatus (model, LC-4A) equipped with visible/ ultraviolet (UV) detector (model, SPD-2AS) at 280 nm and stainless steel column (25.0 cm × 4.6 mm i.d.) (Phenomenex Co., USA) coated with ODS, (RP-18). An aliquot of the sample suspended in MeOH was diluted with 10 mM phosphoric acid buffer (pH 3.5) to the same concentration as initial mobile phase (15% MeOH). Samples were next filtered through a 0.2 µm poly (tetrafluoroethylene) (PTFE) filter prior to injection. The two solvent systems consisted of MeOH (A) and 10 mM phosphoric acid buffer, pH 3.5 (B), operated at following rate of 1.5 ml/min. The phosphoric acid buffer consisted of 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$ adjusted to pH 3.5 with 10 mM H_3PO_4 .

Bacterial and fungal strains

Cultures of seven human pathogenic bacteria, three Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and four Gram-positive (*Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *S. aureus* and *Micrococcus*) bacteria were used for the *in vitro* antibacterial assay. For the antifungal assay, four fungi (*Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger* and *Candida albicans*) were used. The bacterial and fungal strains were obtained from King Abdulaziz University Hospital. The tested organisms were sub-cultured on nutrient agar medium (Oxoid laboratories, UK) slopes for bacteria and Saboroud dextrose agar slopes (Oxoid laboratories, UK) for fungi were the media used. These stock cultures were stored in the dark at 4°C during the survey.

Antimicrobial activity

Antimicrobial activity was determined, using the agar well diffusion assay method as described by Holder and Boyce (1994). DMSO was used as a negative control and streptomycin and ciprofloxacin (10 mg/disc) were used as a positive control for bacteria amphotericin B and nystatin were used as positive control for fungi. The plates were done in triplicate. Bacterial cultures were incubated at 37°C for 24 h while the other fungal cultures were incubated at 30 to 32°C for 48 h. Solution of 10 mg/ml of streptomycin, ciprofloxacin, nystatin and amphotericin B were used as standard for comparison. Antimicrobial was determined by measurement zone of inhibition (Agwa et al., 2000)

Determination of minimum inhibitory concentration (MIC)

The MIC of the extracts was estimated for each of the tested organisms in triplicates. To 0.5 ml of varying concentrations of the extracts (25, 50, 75, 100, 150 and 200 mg/ml), 2 ml of nutrient broth was added and then a loopful of the test organism previously diluted to 0.5 McFarland turbidity standard for all bacterial isolates was introduced to the tubes. A tube containing nutrient broth only was seeded with the test organisms to serve as control. Tubes containing bacteria cultures were then incubated at 37°C for 24 h. The tubes were then examined for bacterial growth by observing for turbidity (Doughari, 2006) (Table 4).

Statistical analysis

For each experiment, three replicates and three determinations were conducted. Means of variable, standard error and least significant differences were carried out using SPSS to detect any significant differences between pathogenic microorganisms and extract type.

Table 1. Chemical composition analysis of *C. equisetifolia* leaves extracts.

Phenol compound (mg/g)	Retention time	<i>C. equisetifolia</i>
Gallic	4.8	19.18
Protocatioic	6.8	6.84
Chlorogenic	9.4	1.63
p.Hydroxy benzoie	10.11	2.58
p.coumaric	11.58	4.70
Syringic	15.07	11.57
Vanillic	18.27	2.88
Salicylic acid	20.15	3.76
Total		53.15

RESULTS and DISCUSSION

Phytochemical screening of *Casuarina equisetifolia* leaves

The phytochemical screening of leaves of *C. equisetifolia* in Table 1 showed that the HPLC analysis successfully provided the presence of 8 phenolic compounds, The major phenol compounds that were isolated from leaves of *C. equisetifolia* were gallic, protocatioic, chlorogenic, p.hydroxy benzoie, p.coumaric, syringic, vanillic and salicylic acid. Gallic has the highest concentration followed by syringic and protocatioic 19.18, 11.57, 6.84 µg/g, respectively. The concentration of other phenolic compounds ranged from 1.63 to 4.70. Chlorogenic compound was the lowest phenol compound 1.63 µg/g. Aher et al. (2010) studied the phytochemical screening of leaves of *C. equisetifolia* and concluded that contain carbohydrate, protien, steroid and tannin in all plant parts. In addition, the phytoconstituents reported were alkaloid (bark), saponin (bark, fruit) and flavonoid (fruit, leaf). These studies confirm the results of Iqbal and Arina (2001) who stated that the main compounds of *C. equisetifolia* leaves extracts were phenols and tannins. These findings correlate with the observations of Tanaka et al. (1991) and Salie et al. (1996). Harisaranraj et al. (2010) reported that the presence of phenols, tannins, steroids, alkaloids, glycosides, flavonoids and terpenoids. Ogunwande et al. (2011) studied the leaf essential oil of *C. equisetifolia* L (Casuarinaceae) comprised mainly of pentadecanal (32.0%) and 1.8-cineole (13.1%), with significant amount of apiole (7.2%), α-phellandrene (7.0%) and α-terpinene (6.9%), while the fruit oil was dominated by caryophyllene-oxide (11.7%), trans- linalool oxide (11.5%), 1.8-cineole (9.7%), α- terpineol (8.8%) and α-pinene (8.5%). The phenolic phytoconstituents isolated from *C. equisetifolia* leaves extracts prompted to evaluate antimicrobial activity and antioxidant potential (Aher et al., 2009). Kishore and Rumana (2012) reported that the presence of carbohydrates, alkaloids, proteins, glycosides, saponins, flavonoids and tannins of *C. equisetifolia* bark extract might be responsible for the

antimicrobial activity and antispasmodic effect (Figure 1).

Extracts of *Casuarina equisetifolia*

The antimicrobial activities of leaves extracts of *C. equisetifolia*, obtained with six different organic solvents and aqueous extracts, the methanol extracts showed the highest activity followed by the ethanol and chloroform extract against the tested bacterial strains (Table 2). Methanol extracts in this study might have had higher solubility for more phytoconstituents, resulting, the highest antibacterial activity. These studies confirm the results of Rajib et al. (2009) and Jigna et al. (2005) who recorded that the tested methanol extract of leaf and bark of *C. equisetifolia* showed good activity against different strains. The demonstration of antibacterial activity by water extracts against *E. coli*, *K. pneumoniae* and *p. aeruginosa* provides the scientific basis for the use of these plants in the traditional treatment of disease. Methanol extract showed best activity against *S. aureus* > MRSA > *Micrococcus* > *B. subtilis*. Among the Gram-positive bacteria, these results of Iqbal and Arina (2001) stated that the sensitivity of test strains was *S. aureus* > *Staphylococcus dysenteriae* > *Staphylococcus paratyphi* > *B. subtilis*. Similar to our results, *B. subtilis* was least sensitive compared to other test bacteria, which may be due to their ability to form highly resistant resting stage called endospores. The methanol extracts of leaves of *C. equisetifolia* were also tested on three Gram-negative bacteria; showed a strong activity against *E. coli*, followed by *K. pneumoniae* and *P. aeruginosa*. The plant extracts in organic solvent provided more consistent antimicrobial activity compared to those extracted in water. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay (Jigna et al., 2005). Similar results have been reported in previous studies (Ahmed et al., 1998). The difference in potency of plant extracts may be due to different sensitivity of the tested strains and methods of

Table 2. The antibacterial activity of aqueous and organic extract of *C. equisetifolia* concentration 100 mg/ml compared to Antibiotics against different pathogenic bacteria.

Types of bacteria	Pathogenic bacteria	Type of the extract							Positive control		The mean of pathogenic bacteria
		Water	Ethanol	Petroleum ether	Diethyl acetate	Ethyl acetate	Chlorofom	Methanol	Antibiotics		
									Streptomycin (10 µg/disc)	Ciprofloxacin (10 µg/disc)	
Gram-positive bacteria	<i>B. subtilis</i>	0.00 ± 0.00	21.33 ± 0.33	18.33 ± 0.33	16.00 ± 0.00	15.33 ± 0.33	21.00 ± 0.33	23.33 ± 0.33	25.00 ± 0.00	34.00 ± 0.00	16.47
	MRSA	0.00 ± 0.00	23.33 ± 0.33	18.00 ± 0.00	19.00 ± 0.58	17.33 ± 0.33	18.67 ± 0.88	25.33 ± 0.33	20.00 ± 0.00	36.00 ± 0.00	17.38
	<i>S. aureus</i>	0.00 ± 0.00	30.33 ± 0.33	13.33 ± 0.33	15.67 ± 0.33	18.00 ± 0.58	23.33 ± 0.33	36.67 ± 0.33	19.00 ± 0.00	38.00 ± 0.00	19.62
	<i>Micrococcus</i>	0.00 ± 0.00	23.00 ± 0.00	13.67 ± 0.00	15.33 ± 0.33	19.00 ± 0.58	21.33 ± 0.33	23.67 ± 0.33	27.00 ± 0.00	46.00 ± 0.00	16.57
Gram-negative bacteria	<i>E. coli</i>	15.33 ± 0.33	26.00 ± 0.58	19.67 ± 0.00	19.67 ± 0.33	17.33 ± 0.33	23.00 ± 0.00	35.00 ± 0.00	23.00 ± 0.00	44.00 ± 0.00	19.47
	<i>K. pneumoniae</i>	17.67 ± 0.67	33.33 ± 0.33	20.33 ± 0.00	22.00 ± 0.00	20.00 ± 0.00	22.33 ± 0.33	34.67 ± 0.33	25.00 ± 0.00	42.00 ± 0.00	24.33
	<i>P. aeruginosa</i>	18.00 ± 0.00	29.33 ± 0.67	20.33 ± 0.00	23.33 ± 0.33	24.00 ± 0.58	24.67 ± 0.33	33.33 ± 0.33	22.00 ± 0.00	42.00 ± 0.00	24.71
Mean of type of the extract		17.00	26.66	17.66	18.71	18.71	22.05	30.28	23.00	40.29	

There are highly significant differences between pathogenic bacteria. There are highly significant differences between type of the extract. p-value = 0.00.

extraction (Nimri et al., 1999). Similar results on the Gram-negative bacteria have been reported in other studies. Rajib et al. (2009) recorded that the methanol extracts showed largest zone of inhibition against *K. pneumoniae* and measured 28 mm followed by *Shigella sonni*. Antimicrobial activities of standard antibiotics showed an inhibitory effect against all the tested bacteria. The result also showed that the ciproflaxocin is more effective than streptomycin. The present results go in line with Shital (2010). The antifungal activity of aqueous and organic extracts of *C. equisetifolia* leaves extracts compared to standard antibiotics are shown in Table 3. The data showed that the ethanol extracts exhibited strong activity followed by methanol extracts against all the tested fungi. The ethanol extract exhibited remarkable antifungal activities against the tested fungi in the order of sensitivity as *A. flavus* > *A. niger* > *A. fumigatus* > *C. albicans*. The lowest sensitive in *C. albicans* of these result agreed with Han (1998)

who reported the antifungal of *C. equisetifolia* leaves extract. The aqueous extracts of *C. equisetifolia* leaves were inactive against all the tested fungi. The antifungal activities of standard antibiotics showed that the amphotericin is less effective than nystatin.

From Tables 2 and 3, drug-resistant strains of bacteria and *C. albicans* were found to be sensitive to the tested plant extracts; this has clearly indicated that antibiotic resistance does not interfere with the antimicrobial action of plant extracts and these extracts might have different modes of action on test organisms (Iqbal and Arina, 2001). Previously, antimicrobial activities of different *C. equisetifolia* parts have been studied by Salem et al. (2011). Thus, our antimicrobial screening result also justifies the traditional uses of these plants in various ailments including infectious disease. Further, the active phytochemicals of these plants against multidrug-resistant bacteria and *C. albicans* has to be

characterized and the efficacy of non-toxic extracts preparations has to be evaluated *in vivo*. The *C. equisetifolia* extracts can be subjected to isolation of the therapeutic, antimicrobials and undergo further pharmacological evaluation.

Mode of antimicrobial action

Results of MICs of leaves extracts of *C. equisetifolia* against tested bacteria organisms are shown in Table 3. The results showed significant difference in their MIC. In case of using Gram-positive bacteria, the MICs were ranged from 50 to 150 µg/ml for *B. subtilis* while they were ranged from 50 to 100 µg/ml for MRSA. On the other hand, they were ranged from 25 to 150 µg/ml in case of *Micrococcus* for organic extracts of *C. equisetifolia* leaves. The MIC for Gram-negative bacteria is shown also in Table 3. The results showed that the MIC were ranged from

Table 3. The antifungal activity of aqueous and organic extract of *C. equisetifolia* concentration 100 mg/ml between Type of the compared to antibiotics against different pathogenic fungi.

Pathogenic fungi	Zone of inhibition (mm)							Positive control		Mean of fungi
	Type of the extract							Antibiotics		
	Water	Ethanol	Petroleum ether	Diethyl acetate	Ethyl acetate	Chlorofom	Methanol	Amphotericin	Nystatin	
<i>A. flavus</i>	0.00 ± 0.00	39.00 ± 0.58	17.00 ± 0.58	14.67 ± 0.33	16.00 ± 0.00	17.67 ± 0.33	21.00 ± 0.58	29.00 ± 0.00	32.00 ± 0.00	17.90
<i>A. fumigatus</i>	0.00 ± 0.00	24.33 ± 0.88	19.00 ± 0.58	19.33 ± 0.33	17.67 ± 0.33	20.33 ± 0.33	22.00 ± 0.58	26.00 ± 0.00	28.00 ± 0.00	17.52
<i>A. niger</i>	0.00 ± 0.00	28.00 ± 0.58	19.67 ± 0.33	14.33 ± 0.33	14.00 ± 0.00	23.00 ± 0.58	17.00 ± 0.58	25.00 ± 0.00	30.00 ± 0.00	16.57
<i>C. albicans</i>	0.00 ± 0.00	22.67 ± 0.33	14.67 ± 0.67	18.00 ± 0.58	18.00 ± 0.00	16.33 ± 0.33	20.00 ± 0.00	28.00 ± 0.00	29.00 ± 0.00	15.67
Mean of type extract	0.00	28.5	17.58	16.58	16.42	19.33	20	27.00	29.75	

There are highly significant differences between pathogenic fungi. There are highly significant differences between type of the extract .F= 64.01; p-value = 0.00.

Table 4. Minimum inhibitory concentration (MIC) of *C. equisetifolia* leaves extracts.

Types of bacteria	Pathogenic bacteria	MIC (Mg/ml)					
		Ethanol	Petroleum ether	Diethyl acetate	Ethyl acetate	Chlorofom	Methanol
Gram-positive bacteria	<i>B. subtilis</i>	50	100	150	150	100	50
	MRSA	50	100	100	100	50	50
	<i>S. aureus</i>	25	150	150	100	50	25
	<i>Micrococcus</i>	50	150	150	100	50	50
Gram-negative bacteria	<i>E. coli</i>	25	50	50	100	50	25
	<i>K. pneumoniae</i>	25	50	50	50	50	25
	<i>P. aeruginosa</i>	50	50	50	50	50	50

25 to 100 µg/ml for *E. coli* while they were ranged from 25 to 50 µg/ml in *K. pneumoniae* and *P. aeruginosa*. In other study, Rajib et al. (2009) reported that the MICs of *C. equisetifolia* were 64 and 128 µg/ml against *S. aureus* and *S. sonnei*, respectively and 256 µg/ml against *B. subtilis*. So, it is evident that the extracts under study showed inhibition of bacterial growth even at low concentrations.

The MIC is lowest for *C. equisetifolia* against both Gram-positive *S. aureus* and Gram-negative *S. sonnei*, respectively. Thus, *C. equisetifolia* are more suitable in aspect of antibacterial activity. This inhibitory effect of the extract on the growth of these microorganisms could be attributed to the presence of some phytochemicals that were found present in the *C. equisetifolia* extract. The demonstration of antibacterial activity against pathogenic

bacteria and fungi may be indicative of the presence of broad spectrum antibiotic compounds (Doughari, 2006). Previously, antimicrobial activities of various plants has been studied by various authors (Nisar et al., 2010a, b, 2011; Qayum et al., 2012; Zia-Ul-Haq et al., 2011).

The optimal effectiveness of the medicinal plant may not be due to one main active constituent, but may be due to the combine action of different

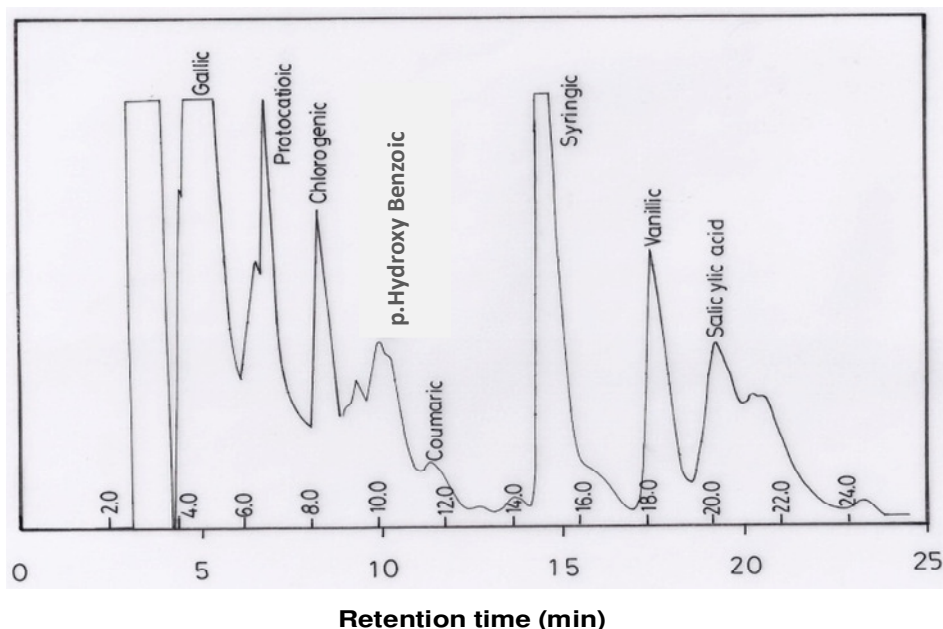


Figure 1. Phenol compound of *C. equisetifolia* leaves extracts.

compounds originally present in the plant (Bai, 1990).

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