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Phytochemical screening of plant extracts and GC-MS analysis of *n*-Hexane soluble part of crude chloroform extract of *Cuscuta reflexa* (Roxb.)

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Abstract
The phytochemical constituent of chloroform and methanol extracts of *Cuscuta reflexa* (whole plant) was identified by phytochemical screening and gas chromatography-mass spectroscopy (GC-MS) techniques. Qualitative analysis of the plant sample revealed the presence of steroids, terpenoids, flavonoids, phenolic compounds and cumarins in both the extracts, but flavonoids, alkaloids, saponines and tannins are present only in methanol extract. The GC-MS analysis of *n*-hexane soluble part of chloroform extract of *C. reflexa* revealed eight major peaks with comparatively higher peak area due to Tetrapentacontane (29.98%), Tris(2,4-di-tert-butylphenyl) phosphite (20.87%), Tris(2,4-di-tert-butylphenyl) phosphate (13.40%), 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (2.66%), 2,4-Di-tert-butylphenol (2.62%), Dotriacontane (2.31%), Eicosane (2.28%) and 4,6-dimethyl-dodecane (2.17%). Overall, the study summarizes the information regarding the presence of pharmaceutically important bioactive constituents.

Keywords: *C. reflexa* (Roxb.), plant extracts, phytochemical constituents, GC-MS analysis

1. Introduction
Medicinal plants are rich in therapeutic properties and thus they are used for different medicinal purposes. Science the ancient time natural products obtained from medicinal plants are being used in this subcontinent for the treatment of various diseases [1]. This indicates that the medicinal plants are storehouse of pharmacologically important compounds and can be used for the treatment of various ailments. Hence, it is important to carry out biological studies on different medicinal plants to identify their appropriate medicinal value. But a vast number of medicinal plants and their phytochemical properties are still unrecognized [2]. The medicinal systems that use medicinal plants are Ayurveda, Unani etc., to cure various diseases. Drugs obtained from natural sources are often found to contain lesser side effects. Hence, in this subcontinent there is a long tradition of using herbal medicines by the native people. Owing to the wide diversity in local plants, herbal medicines are used for primary medication especially in the villages.

Phytochemicals are the non-nutritive plant chemicals which have the properties to prevent or protect various diseases. These chemicals are produced by plants to defend themselves but researches have revealed that the chemicals have the competence to treat human diseases [3]. There are a wide range of phytochemicals, which have distinctive pharmacological properties [4].

Cuscuta reflexa is a parasitic weed plant which is also known as Swarnalata in Bengali and Dodder in English [5]. It sucks nutrient from the host plant to carry out biological activity. Hence the phytochemicals identified largely depends on the host plant [6]. The present study was undertaken to evaluate the medicinal significance of the whole plant of *Cuscuta reflexa.* In this experiment phytochemical screening provides the qualitative analysis while the gas chromatogram-mass spectrometric (GS-MS) method offers the quantitative estimation of the phytochemical constituents of the plant sample.

2. Materials and methods
2.1 Collection of the Plant
The whole plant of *Cuscuta reflexa* was collected from the host plant *Ziziphus mauritiana* (Bengali: Kul) from a north eastern district, Dinajpur, of Bangladesh in January of 2018. The plant materials were then identified from Bangladesh National Herbarium at Dhaka and voucher specimens (specimen no. 43869) were deposited at the herbarium.

2.2 Preparation of Extract
The plant material was cut into small pieces and dried under the shade and then powdered in grinder machine. The plant sample was then extracted with chloroform and methanol successively at room temperature. The chloroform and methanol extracts were used for phytochemical screening and the hexane soluble part of chloroform extract was used for the GC-MS analysis.

2.3 Methodology of Phytochemical Screening
Phytochemical screening of chloroform and methanol extracts of *C. reflexa* was carried out to evaluate the presence of phytochemicals, such as, alkaloids, coumarines, glycosides, [7, 8] flavonoids, tanines, carbohydrates, anthraquinones, saponines etc [9, 10]. Standard chemical procedures were followed for the identification of phytochemical constituents as described by Trease and Evans [11], Harborne [12], and Sofwara [13]. The result of phytochemcial study is presented in Table-1.

2.4 Instrumentation and methodology of GC-MS analysis
The *n*-hexane soluble part of crude chloroform extract of *Cuscuta reflexa* was analyzed by Electron Impact Ionization (EI) method on a GC-17A gas chromatograph attached to a MS 2010 plus mass spectrometer. The temperature (40 °C) of Fused silica capillary column was maintained with the help of carrier gas (helium) at a constant pressure of 90 kPa. The sample was dissolved in chloroform and the range of linear temperature increased 10ºC per min. The operating conditions were as follows: name of column- RTS-5MS, length 0.25 mm, diameter 30cm, temperature of the column-initial temperature 40 °C (for 2 min), injector temperature- 220 °C, holding time 5 min, the column was packed with 10% diethylene glycol succinate on 100-200 mesh diatomic CAW, samples were injected by splitting with split ratio 10, carrier gas (helium) pressure was constant at 90 kPa.

GC-MS analysis allows the identification and quantification of compounds present in the experimental sample. This analysis technique provides a representative spectral output of all the identifiable compounds from the experimental sample. The GC-MS analysis is first initiated by injecting the experimental sample to the injection port of the Gas chromatography (GC) device. The GC equipment evaporates the sample and then separates and investigates different components present in that sample. Each component ideally produces a specific spectral peak that may be recorded on a paper chart electronically.

For the current study, the compounds are identified by using their retention time (time between the elusion and injection). The mass spectrum in GC-MS device was interpreted by using database of National Institute Standard and Technology (NIST) comprising more than 62000 patterns [14]. The spectrum obtained for unknown compounds present in the experimental sample were then compared with that of the known compounds stocked up in the NIST library. The molecular formula, retention time, molecular weight and composition percentage of different compounds present in the sample material of *C. reflexa* were thus recorded and presented in figure-1 and table-2.

3. Result and discussions
3.1 Analysis of Phytochemical Constituents
Phytochemical screening of the chloroform and methanol extract was analyzed qualitatively in the lab by using chemical methods. The presence of steroids, terpenoids, flavonoids, phenolic compounds, coumarines, quinines, alkaloids, saponines, anthraquinones and tannins in the plant extract was tested in this experiment. The phytochemical screening of the chloroform and methanol extracts revealed the presence of steroids, terpenoids, flavonoids, phenolic compounds, cumarins, alkaloids, saponines and tannins.

It is evident from the literature that the therapeutic potency of any important component may possibly be increased by the presence of carbohydrate [15]. It was also found that tannins show antidiarrheal activity and these constituents may precipitate proteins on the enterocytes which reduces peristaltic movement and intestinal secretion [29]. Therefore, better curative outcome can be obtained from the combination of principle constituents present in the plant material than by a single distinct isolated component [16-17]. In addition to that, saponins have hypoglycemic, cardiotonic and expectorant activity [18-21]. Furthermore, glycosides exhibit antiseptic, diuretic and laxative properties [22-24] as well as flavonoids have significant antimicrobial [25], anti-diabetic [26], antioxidant [27], hypoglycemic, anti-inflammatory, anti-tumour [28] and free radical-scavenging activities. The current experiment revealed that the chloroform and methanol extract of *Cuscuta reflexa* may have antioxidant, anti-inflammatory agents. The presence of flavonoids and tannins may exhibit free radical-scavenging and antidiarrheal activity, respectively. In addition to that, the plant sample may also show cytotoxicity due to the presence of terpenoids [28].

Table 1: The results of phytochemical screening of the chloroform and methanol extracts of *C. reflexa* (whole plant)

Tests for	Tests and Observations	Comment (Chloroform extract)	Comment (Methanol extract)
1. Steroids	Leibermann Burchard Test: 10 mg extract (dissolved in $CHCl_3$) + few drops of Ac_2O + H_2SO_4 = formation of green color.	+ ve	+ ve
2. Terpinoids	LeibermannBurchard Test: 10 mg extract (dissolved in $CHCl_3$) + few drops of Ac_2O + H_2SO_4 = formation of pink color.	+ ve	+ ve
3. Flavonoids	Shindo's test: 10 mg extract (dissolved in MeOH) + Mg + few drops of conc. HCl = change of color with bubbling in case of MeOH extract.	- ve	+ ve
4. Phenolic compounds	10 mg extract (dissolved in $CHCl_3$) + 1-2 drops of $FeCl_3$ = formation of red, blue, green or purple color.	+ ve	+ ve
5. Coumarins	10 mg extract (dissolved in MeOH) + alcoholic KOH (few drops) = formation of yellow color.	+ ve	+ ve
6. Quinones	10 mg extract (dissolved in MeOH) + H_2SO_4 = no color development.	- ve	- ve
7. Alkaloids	10 mg extract + Mayer's reagent = formation of cream colored precipitation in case of MeOH extract. Mayer's reagent = $HgCl_2$(.34g)+ KI(1.25g) + H_2O (25ml)	- ve	+ ve
8. Saponines	Frothing test: 10 mg extract + H_2O = formation of stable foam for MeOH extract.	- ve	+ ve

9. Anthraquinone	5 mg extract (dissolved in 5 ml organic reagent) + 10% ammonia solution = no development of bright pink, red or violet color in the upper layer.	- ve	- ve
10. Tannins	5 ml aqueous extract + few drops of 1% lead acetate solution = yellow precipitate found for MeOH extract.	- ve	+ ve

3.2 GC-MS analysis of the plant extract

Identification and quantification of the constituents present in the experimental plant sample was performed by GCMS method. Peak area, molecular formula and retention time were used to confirm the identification of the compounds present in the plant sample. A total of 21 *n*-hexane soluble compounds were identified from the whole plant of *Cuscuta reflexa* by GC-MS analysis, which represents the medicinal quality of the plant sample. The TIC curve and different analyzed values of the *n*-hexane soluble compounds of *C. reflexa* is shown in Figure-1 and Table-2.

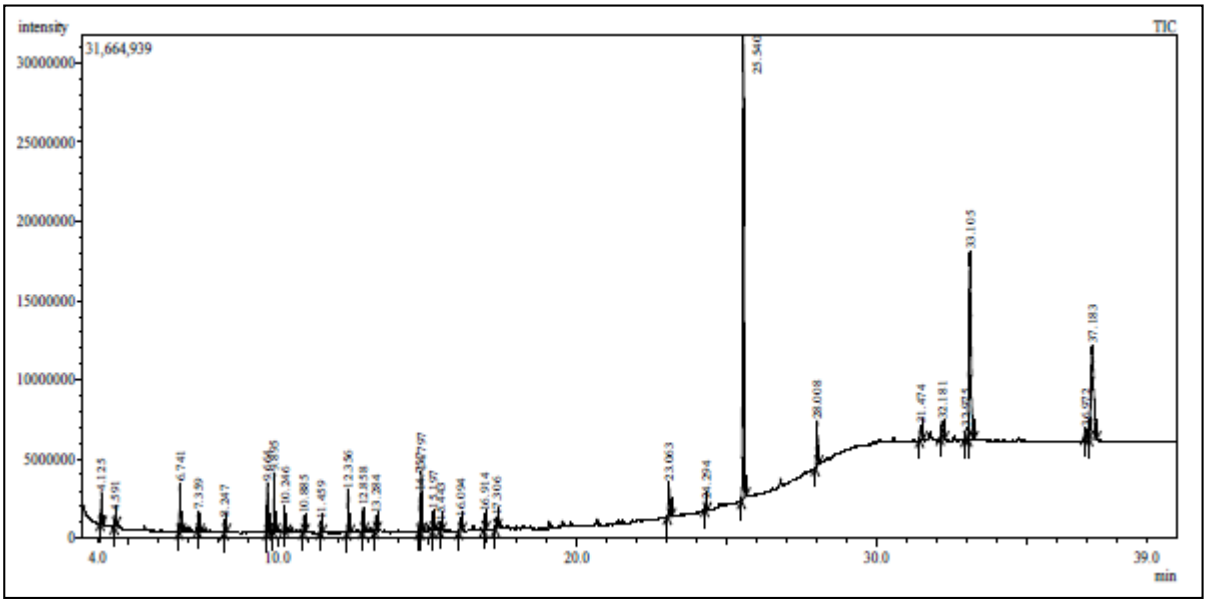


Fig 1: TIC of the *n*-hexane soluble part of crude chloroform extract of *C. reflexa* (GC-MS)

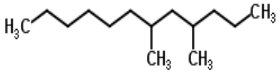
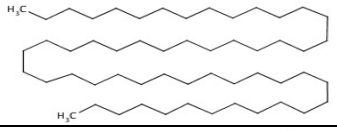
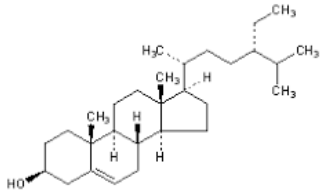
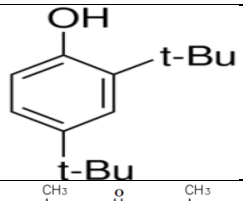
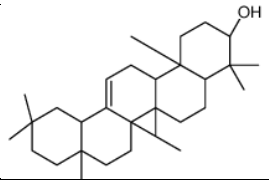
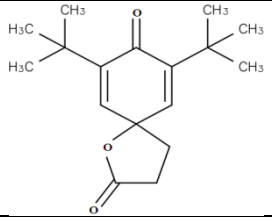
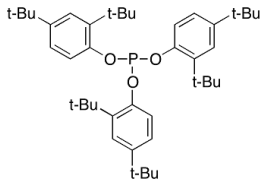
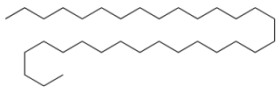
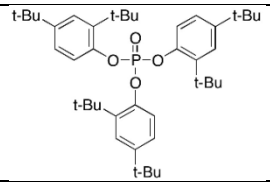
Table 2: GC-MS analysis of the *n*-hexane soluble part of crude chloroform extract of *C. reflexa*

Sample No	Retention time	Name of the Compound	Molecular weight	Molecular formula	Class of compound	Conc. (%)
NH-1	4.125	5-(2-methylpropyl) nonane	184.367	$C_{13}H_{28}$	Alkane	1.15
NH-2	4.591	6-ethyl-2-methyl Octane	156.313	$C_{11}H_{24}$	Alkane	0.56
NH-3	6.741	4,6-dimethyl odecane	198.388	$C_{14}H_{30}$	Alkane	2.17
NH-4	7.359	4-Methyldodecane	184.367	$C_{13}H_{28}$	Alkane	0.96
NH-5	8.247	1-Pentadecene	210.405	$C_{15}H_{30}$	Alkene	0.64
NH-6	9.664	Eicosane	282.556	$C_{20}H_{42}$	Alkane	2.28
NH-7	9.895	2,4-Di-tert-butyl Phenol	206.329	$C_{14}H_{22}O$	Phenol	2.62
NH-8	10.885	1-Tetradecanol	214.393	$C_{14}H_{30}O$	Fatty alcohol	0.72
NH-9	11.459	2,6-di-tert-butyl-4-hylidenecyclohexa-2,5-dien-1-one	232.367	$C_{16}H_{24}O$	α, β - unsaturated ketone	0.51
NH-10	13.284	1-Nonadecene	266.513	$C_{19}H_{38}$	Alkene	0.62
NH-11	14.797	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	276.376	$C_{17}H_{24}O_3$	α, β - unsaturated ketone	2.66
NH-12	15.443	1-Heptacosanol	396.744	$C_{27}H_{56}O$	Fatty alcohol	0.36
NH-13	16.094	3,5-di-tert-Butyl-4-hydroxyanisole	236.349	$C_{15}H_{24}O_2$	Phenolic compound	0.78
NH-14	23.063	Dotriacontane	450.880	$C_{32}H_{66}$	Alkane	2.31
NH-15	25.540	Tetrapentacontane	759.474	$C_{54}H_{110}$	Alkane	29.98
NH-16	31.474	γ-Sitosterol	414.718	$C_{29}H_{50}O$	Steroid	1.38
NH-17	32.181	β-Amyrin acetate	468.754	$C_{32}H_{52}O_2$	Triterpenoid	1.67
NH-18	32.975	Lupeol	626.729	$C_{30}H_{50}O$	Triterpenoid	1.22
NH-19	33.105	Tris(2,4-di-tert-butylphenyl) Phosphite	646.922	$C_{42}H_{63}O_3P$	Phosphite ester	20.87
NH-20	36.972	Octadecyl 3-(3,5-di- tert-butyl-4-hydroxyphenyl) propionate	530.865	$C_{35}H_{62}O_3$	Ester	1.04
NH-21	37.183	Tris(2,4-di-tert-butylphenyl) Phosphate	662.936	$C_{42}H_{63}O_4P$	Phosphate ester	13.40

In case of the *n*-hexane soluble part of chloroform extract of *C. reflexa*, it can be seen from both Table-2 and Figure-1 that the total amount of identified compound was 87.90% and the composition of nearly 12.1% remained unidentified. Here major components were identified as Tetrapentacontane (29.98%), Tris(2,4-di-tert-butylphenyl) phosphite (20.87%), Tris(2,4-di-tert-butylphenyl) phosphate (13.40%), 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (2.66%), 2,4-

Di-tert-butylphenol (2.62%), Dotriacontane (2.31%), Eicosane (2.28%) and 4,6-dimethyldodecane (2.17%), these eight compounds together comprised 76.29% of the fraction. The concentration of the following compounds were in between 0.36-1.67%: β-Amyrin acetate (1.67%), 5-(2-methylpropyl) nonane (1.55%), γ-Sitosterol (1.38%), Lupeol (1.22%), Octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate (1.04%), 4-Methyldodecane (0.96%), 3,5-di-tert-Butyl-4-hydroxyanisole (0.78%), 1-Tetradecanol (0.72%), 1-Pentadecene (0.64%), 1-Nonadecene (0.62%), 6-ethyl-2-methyloctane (0.56%), 2,6-di-tert-butyl-4-ethylidenecyclohexa- 2,5-dien-1-one (0.51%), 1-Heptacosanol (0.36%).

Table 3: Structure of some major components identified from the hexane extract of *C. reflexa* are shown in the Table-3

Name and Sample No. of the Compound	Chemical structure	Name of the compound	Chemical structure
4,6-dimethyl dodecane (NH-3)		Tetrapentacontane (NH-15)	
Eicosane (NH-6)		γ-Sitosterol (NH-16)	
2,4-Di-tert-butylphenol (NH-7)		β-Amyrin acetate (NH-17)	
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (NH-11)		Tris(2,4-di-tert-butylphenyl) Phosphite (NH-19)	
Dotriacontane (NH-14)		Tris(2,4-di-tert-butylphenyl) phosphate (NH-21)	

4. Conclusion

The present study provides an overview of the presence of large number of secondary metabolites in the whole plant of *Cuscuta reflexa* and indicates the presence of different class of compounds that may show pharmacological importance. Thus, further studies are required on this plant material to isolate as well as elucidate the structure of the bioactive compounds.

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