Research Article

ISOLATION AND CHARACTERIZATION OF FLAVONOID COMPOUND EXTRACTED FROM *CARDANTHERA DIFFORMIS* DRUCE AND STUDY OF ITS ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES *IN VITRO*

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ABSTRACT

Human beings have been utilizing plants for basic preventive and curative health care since time immemorial. Medicinal plants have been used to treat illness and disease for thousands of years. Even now they are economically important, being used in the pharmaceutical, cosmetic, perfumery, and food industries. Screening of medicinal plants for antimicrobial activities and phytochemicals is important for finding potential new compounds for therapeutic use. Humans are now struggling for their existence against different oxidative stress related diseases. Different meta-analysis suggests that poly-phenols, especially flavonoids, may play best protective role against this epidemiology. In this paper, an attempt has been made to present the results of the experimental studies carried out to characterize a flavonoid compound that has been isolated and primarily identified from a plant extract of *Cardanthera difformis* Druce. The compound has similar physiochemical properties like quercetin. Antibacterial activity of extracted flavonoid has been carried out against various types of bacterial human pathogens. The result shows that the extract of *Cardanthera difformis* has a good anti-bacterial activity against *Salmonella typhi*. It has been found that this anti-bacterial flavonoid compound is good anti-oxidant compound and the anti-oxidant potentiality of the same has been quantified by DPPH-spectrophotometric assay.

Keywords: *Cardanthera difformis*, flavonoid, antibacterial activity, anti-oxidant activity, DPPH-spectrophotometric assay.

INTRODUCTION

Polyphenols have potential antioxidant activity and can play protective role against oxidative-stress induced different human diseases like type-II-diabetes, cardiovascular diseases, some cancers, etc.¹⁻⁵. Among different polyphenols, flavonoids are most important and are produced as plants secondary metabolites⁶. Vegetables, fruits, flowers, stems, roots, leaves are thus rich storage and source of flavonoids⁷. According to 'Phenol-Explorer' (an online database of polyphenols present in food) different citrus fruits, onions, broccoli, tea, grapefruit, Cocoa beans, red wine are rich in flavonols⁸⁻¹⁰. Different meta-analysis suggests, human should intake polyphenols in their daily dietary food for these essential surrogate nutrients for next generation survival^{11, 12}.

Epidemiologic studies on plant polyphenols are now being very essential as shortage of reliable data. Though different meta-analysis shows protective effects of different flavonoids but their exposure assessment is still required in this highly polluting environment. These antioxidant and anti-pathogenic bioactive-flavonoids are very reliable because they can activate phase-II metabolism and produce sulfated, methoxylated and glucuronidated compounds which have free radical scavenging activities, can induce apoptosis, inhibit cell proliferation and ageing, regulate nitric oxide, exhibit phytoestrogenic activity and so on^{13, 14}.

Bio-flavonoids are classified as favonols, flavan-3-ols, flavones, isoflavones, flavanones and anthocyanins but all are collectively known as Vitamin P. More than 2000 flavonoids are reported in the world and those have been isolated from many plant species like *Lycium barbarum*, *Passiflora plamer*, *Cassia angustifolia*, *Jatropha curcas L* and *Quercus infectoria*, etc. Recently, there has been a growing interest in the investigation and introduction of medicinal plants with various biological activities to the pharmaceutical industries since synthetic drugs have been associated with several side effects on human health. Furthermore, microorganisms indicated a resistance to synthetic antimicrobial agents, which is a serious and immediate concern^{15, 16}. Due to these facts, the exploration of new alternative medicines derived from plants is required. Flavonoids are classified under phenolic groups in plants which have been known to possess antimicrobial activity¹⁷. The mechanisms of flavonoids that are antimicrobial can be classified as the inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism¹⁸.

In this experimental study, an attempt has been made to isolate and characterize flavonoid from a common weed *Cardanthera difformis*. Here we have also studied that flavonoid's antioxidant and anti-bacterial activity against some human pathogens.

MATERIALS AND METHODS

Isolation of plant

Some whole plant samples of *Cardanthera difformis* were collected from local areas of Paschim Medinipur (Latitude- 22°25'00" to 22°57'00" north, Longitude- 87°11' east, Altitude- 23 meters from mean sea level), West Bengal, India. Then those plant samples were air-dried in shadow for 7 days, crushed into small pieces using mortar and pestle. Those crushed pieces were finally powdered in an electric grinder and the powder was stored for flavonoid extraction in future.

Flavonoid extraction

Initially, 100 gm of whole plant powder of *Cardanthera difformis* was immersed in 500 ml ethanol (HPLC grade; Merck, India) for 24 hours at room temperature on a magnetic stirrer^{19,20}. The mixture was filtered using Whatman No. 1 filter paper. The processes were repeated with the remaining residue and 300 ml ethanol and two filtrates were mixed properly. 100 ml 1%-lead acetate solution was added to that filtrate, stirred for 4 hours for complete precipitation, filtered and filtrate was taken. A mixture of 250 ml acetone and 30 ml hydrochloric acid (Merck, India) was taken and mixed with the filtrate. Total mixture solution was stored in 4°C. Every time deep brown colored precipitate was used for different experimental assays after complete evaporation of 100 ml liquid sample by using a rotary evaporator under reduced pressure.

Chemical screening of flavonoids

To detect the presence of flavonoids and phenolic compounds in the plant extract, every time 5 ml of plant extract was separately added with few drops of 1%-potassium hydroxide (KOH) and 1%- ferric chloride (FeCl₃. 2H₂O) solutions. The presence of flavonoids and phenolic compounds in the plant extract were confirmed by the appearance of yellow and blue-green colors, respectively^{21,22}.

Determination of total flavonoid content in the extracts

The total flavonoid content was determined spectrophotometrically according to Ordon ez et al ²³. Briefly, 0.5mL of 1%- aluminum chloride (AlCl₃) ethanol was mixed with the same volume of plant extract. Absorption readings at 415 nm were taken after 30 min against a blank (ethanol). The total flavonoid content was determined using a standard curve with quercetin (0 - 50 µg/L).

Radical-scavenging effect of extracts in DPPH radicals

The free radical scavenging activity of the plant extract was measured according to Adedapo et al. (2008) with some modifications ¹³. 1 mL of the methanolic solution of plant extract (200, 100, 80, 40, 20, 10, and 5 µg/mL) was added to 2

mL 0.04 mM solution of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radicals in methanol. The mixtures were shaken vigorously and allowed to stand for 30 min at room temperature. The absorbance (Abs_{sample}) of the resulting solution was measured at 517 nm and converted into percentage of antioxidant activity (AA) using the following formula: $AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) - 100] = Abs_{control}\}$. Methanol (2.0 mL) and plant extract (1.0 mL) solution was used as the blank (Abs_{blank}). DPPH (2.0 mL) and methanol (1.0 mL) solution was used as the control (Abs_{control}). Ascorbic acid was used as standards.

Identification of the compound

The plant extract was dissolved in methanol and spotted separately with the standard quercetin on a 5 × 10 cm Thin Layer Chromatography (TLC) plate coated with silica gel (TLC Silicagel 60 F254; Merck KGaA, Millipore Corporation, Darmstadt, Germany). 1-Butanol, glacial acetic acid and water were used in 70:25:5 ratios (v/v/v) as the mobile phase and retardation factor (R_f value) was measured after 1 hr from the air dried plate. The spots were collected from the plates and dissolved in methanol (HPLC grade, HIMEDIA)²⁴. The solution was the analyzed separately by using UV-Visible spectrophotometer (Perkin Elmer, λ35-spectrophotometer) and FT-IR spectrophotometer (SIMADZU, Japan, Model No. 8400S)²⁵. This purified material was also subjected to HPLC studies (Mobile phase: Acetonitrile: methanol: glacial acetic acid 70:30:0.1, column C₁₈ 5mm x 150mm, flow rate: 0.5/min, detection: UV detector at 306nm)²⁶.

Antibacterial activity screening

Antibacterial activity of isolated flavonoid extract from *C. difformis* was investigated against some bacterial human pathogens *Bacillus subtilis*, *Escherichia coli*, *Enterobacter faecalis*, *Klebsiella pneumonia* *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Shigella dysenteriae* and *Salmonella typhi* by Agar Well Diffusion method under aseptic condition ¹⁴. The antibacterial screening has been evaluated by measuring the zone of inhibition. The experiment was done in triplicate and the mean diameters of the inhibition zone have been calculated.

RESULTS

Physiochemical properties of the isolated compound

The compound has been isolated from whole plant extract of *Cardanthera difformis* using ethanol. The extract has a deep brown colour with crystal-like appearance with good yield of 0.02gm/dry material. The compound shows positive results of flavonoid and the presence of phenolic -OH group, presence of un-saturation, and carbonyl group (Figure 1). Some physical and chemical characteristics of the compound have been shown in the Table 1.

Figure 1: Functional group tests. (a) Yellow precipitation during flavonoid test, (b) presence of unsaturation and (c) presence of phenolic –OH group

Table 1: Physical and chemical characteristics of the isolated compound

Test	Flavonoid
Description	Red-brown crystals
Melting point	137°C
Solubility test	Soluble in ethanol, methanol, ethyl acetate, acetone, while insoluble in water
Yield (%)	0.02% / 100gm dry weight

Preliminary identification of the isolated compound

During TLC-separation we have found only one spot of 0.82 R_f value (Figure 2 and Table 2). The compound shows maximum absorption at 217 nm with a smaller at 332 nm (Figure 3). FT-IR analysis reveals that the compound contains multi-functional groups (Figure 4) like phenolic –OH, primary amine (–NH₂), carbonyl (–C=O), olefinic C=C and so on (Table 3)

Table 2: Thin Layer Chromatographic separation of the isolated compound

Developing solvent	Test procedure	Spot(s)	R_f value
Butanol: acetic acid: water (70:25:5; v/v/v)	Naked eye	1	0.82(yellow)
Butanol: acetic acid: water (70:25:5; v/v/v)	UV (360 nm)	1	0.82(fluorescent)
Butanol: acetic acid: water (70:25:5; v/v/v)	Iodine and ammonia vapors	1	0.82(brown)

Figure 2: TLC chromatogram of isolated compound (sample) and quercetin (taken as standard)

Figure 3: UV-vis spectra of standard quercetin and test sample

Figure 4: FT-IR analysis of standard quercetin and test sample

Table 3: The functional groups of the purified flavonoid compound from IR-spectrum

Wave number (in cm^{-1})	Band shape	Band	Functional group
3430	Band	O-H	Stretching of phenolic -OH
2930	Sharp	C-H	Aliphatic stretch
1720-1660	Sharp	C=O	Stretching 'C=O' of -CHO group
1452	Sharp	C=C	Stretching of olefinic C=C
1382	Sharp	O-H	Bending of phenolic -OH
1262, 1164	Sharp	C-O	Stretching of 'C-O'

Estimation of flavonoid using HPLC technique

Estimations of flavonoid was done by using Knauer/Germany High Performance Liquid Chromatography (HPLC) in which identifications were made by comprise of retention time obtained at identical chromatographic conditions of analyzed samples and authentic standards (Figure 5,6).

Figure 5: HPLC Chromatogram of Standard quercetin

Figure 6: HPLC Chromatogram of isolated from test sample.

Antioxidant and Antibacterial activity of isolated pure flavonoid compound

DPPH radicals scavenging activity of pure flavonoid compound isolated from whole plant extract of *Cardanthera difformis* and ascorbic acid as a standard results shown in (Figure 7). The isolated compound has 99.93 $\mu\text{g/mL}$ flavonoid concentrations

with high free radical scavenging and antibacterial activities. The compound shows high antibacterial activity against *Staphylococcus aureus*, *Salmonella typhi* and *Klebsiella pneumonia*. The compound has comparatively low antibacterial activity against *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Bacillus subtilis* (Figure 8).

Figure 7: DPPH radicals scavenging activity of pure flavonoid compound isolated from whole plant extract of *Cardanthera difformis* and ascorbic acid as a standard

Figure 8: Graphical representation of Antibacterial activity of Agar well diffusion method by standard quercetin and isolated pure flavonoid compounds from *Cardanthera difformis* against human enteric pathogens

DISCUSSION

Above experimental results clearly show an idea of the unknown flavonoid compound. TLC chromatogram (Figure 2) reveals the description of this flavonoid compound comparing standard flavonoid 'quercetin'. The R_f values of this flavonoid compound and quercetin are same (R_f value= 0.82). So it may be assume that the unknown compound a flavonoid or may be derivative of flavonoid but further purification and separation are essential for complete identification of the isolated compound. The UV-visible spectrum (Figure 3), shows two peaks of maximum absorption at (217 nm) and (375 nm) which is identical to the structure of quercetin. So the isolated plant product compound may be quercetin.

Form IR-spectrum (Figure 4 and table 3), also indicate that the isolated unknown compound is same as standard. We can conclude that the isolated compound has aromatic structure contain phenolic hydroxyl groups and carbonyl groups within its structure. Flavonoids belong to the polyphenolic molecules containing 15 carbon atoms. Flavonoids belong to the polyphenol family. Flavonoids can be visualized as two benzene rings which are joined together with a short three carbon chain. One of the carbon of the short chain is always connected to a carbon of one of the benzene rings, either directly or through an oxygen bridge, thereby forming a third middle ring, which can be five or six member. The Flavonoids consist of 6 major subgroups: chalcone, flavone, flavanol, anthocyanins and iso-flavonoids. Together with carotenes, Flavonoids are also responsible for the colouring of fruits, vegetables and herbs.

From HPLC analysis (Figure 5 and 6), clearly shows that the retention time of standard is equal to the isolated sample, that why we can concluded that the isolated unknown flavonoid is a quercetin molecule. (Figure 7,8), reveal the good results of antibacterial and antioxidant activity of a flavonoid compound from the whole plant extracts of *Cardanthera difformis*.

CONCLUSION

The extract of *Cardanthera difformis* has potential as antibacterial agent. This finding provides an insight into the usage of the whole plant extract of *Cardanthera difformis* in traditional treatment of human enteric pathogen associated bacterial infections as it has high potential inhibition activity for these pathogens. From this study, we can conclude that isolated flavonoid from these plants done a significant role to inhibit the human enteric pathogen. This may also lead to the development of new generation of drugs that possess both chemotherapeutic and chemo-preventative properties which can results in ways of combating the serious problems of diseases.

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