Applications of fern *Dipteris conjugate* in anti-bacterial and anti-lipolytic purpose

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Abstract— The aim of the present study was to assess the biological properties of *Dipteris conjugate* and to identify its functional compounds. The methanolic leaves-extract (MLE) of *D. conjugate* at different concentrations 5 to 30 µg/ml were assessed for anti-bacterial activities by measuring inhibition zones against a panel of pathogenic bacterial strains using agar diffusion method. MLE at a concentration of 25 µg/ml showed marked anti-bacterial activity against all bacterial strains (11-25 mm zone of inhibition) and was maximum against Staphylococcus aureus (25 mm). The MLE of had the best MIC values of 2.25 and 9.0 mg/ml against S.aureus and Enterobacter sp., respe *D. conjugate* actively. The MLE also possessed good anti-lipolytic activity (55%) against a porcine pancreatic lipase (PPL) and cholesterol oxidase inhibition (69%).The present study provided strong experimental evidences that the MLE of *D.conjugate* is not only a potent source of natural anti-oxidants and anti-bacterial activity but also possesses efficient cholesterol degradation and anti-lipolytic activities that might be beneficial in the body weight management. The results suggest that MLE of *D. conjugate* could potentially be employed in traditional medicine as they are rich in compounds with anti-oxidant, anti-microbial and anti-lipolytic properties.

Keywords— Dipteris conjugate; phyto-chemicals; anti-oxidant; anti-bacterial activity.

1. INTRODUCTION

Dipteris conjugate is a primitive fern found in many remote parts of the world, including Britain. In Malaysia and the surrounding countries, it is found mainly in forest clearings at an elevation of 300-2,900 metres. The common name of the *D. conjugate* is umbrella fern because their fronds are divided to the base into two spreading fan-shaped halves, each divided more than half way into 4 unequal lobes, these lobes are again less deeply lobed once or more times[1,2]. The phytochemicals present in this fern offer us the safer natural products that can be developed in the form of therapeutics. These bioactive compounds can be extracted and purified in many ways [3]. Plant-based medicines cover an important portion in current pharmaceutics that we are using these days [4,5] Amongst many phyto-chemical compounds discovered till date for pharmaceutical values, the anti nutrients are among them [6] Phyto-chemicals like rutin and visoltricine are useful in pharmacological treatments. The phyto-chemical screening of fern revealed the presence of alkaloids, saponins, tannins and polyphenols [7]. Fern leaves are known for their anti-microbial activity against many microorganisms. For thousands of years, this fern is considered to have health-giving properties and this has been amply confirmed in recent years [8]. However, other components of umbrella fern, notably, the polyphenols may also contribute to the effects of this fern in view of their known pharmacological properties. The complex of oxidized polyphenols in this fern is often called tannin [9].Some clinical studies have revealed physiological responses to fern extracts that might be relevant to promote health as well as the prevention or treatment of these chronic diseases. Furthermore, inconsistencies among some studies should be resolved by improved approaches to their evaluation [10]. In the present study, the MLE of fern was made and used to assay its biochemical constituents, anti-oxidant/free radical scavenging activities and hemolytic activity besides anti-bacterial activity against a panel of common human pathogenic bacterial strains.

2. MATERIALS AND METHODS

A. Preparation of alcoholic leaves extract of D. conjugate

Leaves of *D.conjugate* were washed under running tap water followed by two washes with distilled water. The leaves were dried in shade, homogenized to a fine powder that was stored in an airtight brown coloured bottle. Ten grams of these powdered leaves were soaked in 100 ml of methanol and the suspension was left at 25^{o}C for 24 h so that alkaloids, terpenoids and other constituents if present in Green tea leaves get dissolved. The methanolic leaves-extract rendered sterile by filtration through 0.22µm membrane filter was assayed for the protein content (Bradford 1976) using BSA as a reference protein. The sterile alcoholic leaf-extract was stored at 4^{o}C in airtight glass vials for further studies.

B. Test microorganism

A total of 12 common pathogenic bacterial strains (Gram negative bacteria: Salmonella typhi, Salmonella paratyphi, Proteus mirabilis, Salmonella typhimurium, Escherichia coli, Shigella flexneri, Pseudomonas aeruginosa, Enterobacter sp., Klebsiella pneumonia and Gram positive bacteria: Streptococcus mutans, Staphylococcus epidermidis and Staphylococcus aureus) were used in the present study to test the MLE of fern for its anti-bacterial, anti-lipolytic, cholesterol oxidase modulation and cytotoxicity towards a mammalian cell line. The bacterial cultures were maintained in viable states by periodic (15 d) sub-culturing on Muller-Hinton(MH) medium (pH 7.2). The MH agar medium (HiMedia, Mumbai, India) prepared in distilled water was sterilized by autoclaving and poured in the petri plates (10 cm diameter) for assay of antibacterial activity of the MLE against the selected pathogenic bacterial strains.

C. Well-diffusion assay for anti-bacterial activity of MLE

Antibacterial activities of *D.conjugate* MLE were tested using well-diffusion method. The Petri plates containing MH-agar based medium were inoculated with selected bacterial strains using the streak plate method. Wells were made on the agar surface with a sterile cork borer (4 mm diameter) aseptically. The MLE was poured into the designated wells using sterile auto-pipette tips. The Petri plates were incubated at 37ºC± 0.5ºC for 24 h for the bacterial growth to appear. The plates were observed in the zone of clearance/inhibition around the wells. The zones of inhibition(s) zone around the well (in mm) including the well diameter were recorded. The observations were taken around the well(s) in three different directions in all 3 replicates and the average values were tabulated.

D. DPPH free radicals scavenging assay

Antioxidants react with 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free radicals to produce its reduced form (DPPH-H). The DPPHH formation in the reaction mixture is assayed at A_{517}.The reduction of DPPH radical to the DPPH-H form is indicated by the formation of a yellow colour. A lower A_{517} of the reaction mixture indicated a higher free radical scavenging activity. Solution of DPPH (0.1 mM) in methanol was prepared and 1.0 ml of this solution was added to 3.0 ml of MLE in methanol at different concentration and 30 minutes later the A517 values were recorded. A blank was prepared without adding MLE to serve as a control. Ascorbic acid (1 to18 µg/ml) was used as a standard. The capability to scavenge the DPPH radicals was determined as follows;

$$DPPH\ scavenged\ (\%) = \frac{A517\ control - A517\ test}{A517\ control} \times 100 \quad (1)$$

E. Assay for cholesterol oxidase activity

A previously reported colorimetric method [11] was used to assay the cholesterol oxidase activity in the MLE. Approximately diluted commercial cholesterol oxidase activity in the MLE. Approximately diluted commercial grade bacterial cholesterol oxidase [Sigma Chemical Co., Saint Louis, USA) was employed to calibrate a reference profile using cholesterol as a

substrate. One unit (U) of cholesterol oxidase activity was defined as the amount of enzyme capable of converting 1.0 µmole of cholesterol to 4-cholesten-3-one per minute at pH 7.5± 0.1 and at a temperature of 37 ± 1 ºC.

F. *MIC of MLE of D.conjugate against selected bacterial strains*

The MIC assay was performed in a 96-well micro-titre plate. For MIC assay, twelve wells in each of the rows of micro-titer plate were used out of which last two wells were taken as control (no MLE was added). Each of the 10 wells received 100 µl of the MH broth; except the 1st well that received 200 µl of broth containing 500 µg/ml of the ALE. From the 1st well, 100 µl of the MH-broth containing MLE was withdrawn with a sterile tip, and the same was added to the 100 µl of the broth taken in the 2nd well, contents were mixed 4 times, then 100 µl of MH-broth was withdrawn from 2nd well and was added to the 3rd well. This way a range of 2-fold serial dilutions was prepared. The MH broth in each of the wells was inoculated with 2 µl of the pure bacterial culture and the content were mixed by 10 clockwise and 10 anti-clockwise rotations on a flat surface. The micro-titer plate was incubated at 37^{o}C for 24 h thereafter the observations for growth of bacteria were visually made and MIC of MLE for each of the test bacteria were recorded and expressed as µg/ml of MLE.

G. Anti-lipase assay

To 2.9 ml of Tris-HCl buffer (0.1 M, pH 8.5) added 80 µl of MLE of fern, incubated the reaction mixture at 37^{o}C in a water bath for 10 min in order to remove the turbidity and added 80 µl of the substrate (p-NPP, 20 mM) along with 20 µl of PPL. The reaction mixture was re-incubated at 55^{o}C in a water-bath for 10 min. The reaction was stopped by chilling at -40^{o}C.The amount of pnitrophenol (p-NP) released was measured at A_{410} (Perkin Elmer UV/VIS Spectrophotometer Lambda 12) after bringing the tubes to room temperature. A standard curve of p-NP was plotted at the selected concentration (10-100 µg/ml) vs. observed A_{410} values.

H. Human RBCs hemolysis test

RBCs in the human blood (Blood group 'O' Rh^{+}) taken in 10% Citrate phosphate dextrose buffer (pH 7.3) were harvested by centrifugation (1,000 g for 5 min at 4^{o}C). The packed RBCs were washed 5-times with Phosphate buffered saline (PBS; 0.05 M, pH 7.3). The packed RBCs were used to make a 0.4% (v/v) suspension in PBS. RBCs suspension (100 µl) was transferred to each of the wells of a 96 U-wells microtiter plate and mixed with (100 µl) of appropriately diluted MLE followed by incubation at 37°C for 1 h. The supernatant (100µl) was transferred to new wells and A_{414} were measured to monitor RBC lysis. RBCs in PBS alone acted as a negative control and RBCs lysed with Triton X-100 (0.1%, v/v) were used to measure 100% lysis (a positive control). The hemolysis percentage was determined as followed;

$$\text{Hemolysis (\%)} = \frac{A_{414}\text{ control} - A_{414}\text{ sample}}{\text{A control}} \times 100$$

3. RESULTS AND DISCUSSION

The present investigation was carried out on leaves of *D.conjugate* to study the presence of biologically active phytochemicals in the fern and to determine its antibacterial, the fat-reducing (anti-lipase),cholesterol degrading (cholesterol oxidase) and DPPH free radical scavenging activities of this fern MLE besides its hemolytic activity.

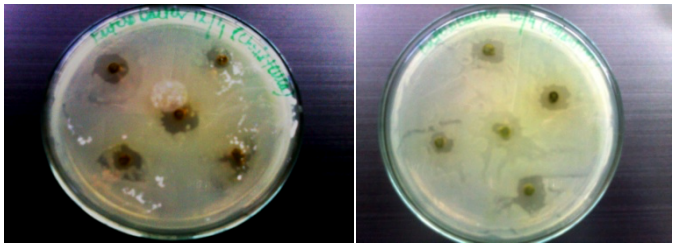
A. Anti-bacterial activity

The present study showed marked antibacterial activity of *D.conjugate* (MLE) against 12 different strains of pathogenic Gram +ve and Gram -ve bacteria (Table 1).

Table1. Anti-bacterial activity of MLE of *D.conjugata* against selected microorganisms.

Organism(s)	**Inhibition zone (Mean value in mm± SD)**
Pseudomonas aeruginosa	20.0± 1.0
Shigellaflexneri Escherichia coli	23.0± 2.7 20.0± 0.5
Salmonella typhi murium	18.0± 1.0
Salmonella paratyphi	23.0± 2.9
Salmonella typhi	23.0± 1.5
Enterobacter sp	22.0± 1.5
Klebssiella pneumoniae	23.0± 1.9
Staphylococcus aureus	25.0± 0.5
Streptococcus mutans	22.0± 1.0
Staphylococcus citreus	22.0± 1.0
Staphylococcus epidermidis	24.0± 0.5

A: Inhibition zones against growth of *S. aureus*
B: Inhibition zones against growth *E. coli*.



C: Inhibition zones against growth of *S.epidermidis*
D: Inhibition zone against growth of *Enterobacter sp.*

Figure 1: Anti-bacterial activity of MLE of a *D.conjugata* against selected pathogenic bacteria

The MLE of *D.conjugate* showed excellent activity against S. aureus with the an average zone of inhibition of 25± 0.5 mm and *S. epidermidis* (24± 0.5 mm) followed by marked antibacterial activities against *Salmonella paratyphi*, *Salmonella typhi* and *Enterobacter* sp. with zone of inhibition of 23.0± 2.9, 23.0± 1.5, and 22.0± 1.5 mm, respectively (Figure1: A-D). The least anti bacterial activity of MLE was recorded against *Salmonella typhimurium* (18.0± 1.0 mm) DPPH free radicals scavenging activity of MLE

B. DPPH free radicals scavenging activity of MLE

The MLE *D.conjugate* of scavenged 73.7%of the DPPH in an in vitro assay. The DPPH test provides information on the reactivity of compounds with a stable free radical DPPH that gives a strong absorption band at 517 nm in the visible region of the spectrum. When the odd electron becomes paired off in the presence of a free radical scavenger, the

absorption reduces and the DPPH solution was decolorized and its color changed from deep violet to light yellow. The degree of reduction in absorbance is reflective of the radical scavenging (anti-oxidant) power of the test (MLE) compound(s).

C. MIC as a measure of antibacterial activity of MLE

The MIC values of MLE of *D.conjugata* were recorded against selected common pathogenic bacteria (Table 1). The MLE of *D.conjugata* had the best MIC values against *S. aureus* and *Enterobacter sp*. (2.25 mg/ml; Table 2).

D Hemolytic activity

Hemolytic activity of *D.conjugate* was found to be 17.6% at 6.25 µg/mL of MLE. Hemolysis assay of MLE was done using human blood of group 'O' Rh+ [12].

E. Anti-lipolytic assay and cholesterol oxidase activity of MLE

Lipase activity was assayed by the method of Winkler and Stuckmann, 1979 by measuring the micromoles of p-nitrophenol released from pnitrophenyl palmitate. The MLE of *D.conjugate* was found to possess 56.5% anti-lipolytic activity against PPL and 69.0% cholesterol oxidase activity.

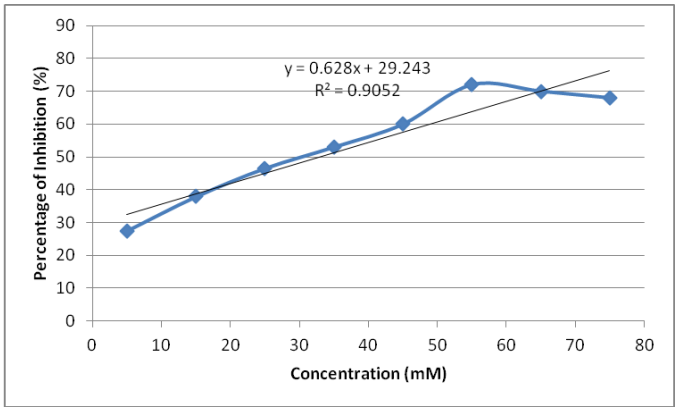


Figure 2: DPPH activity of MLE of a *D.conjugate*

Table 2. MICof MLE of *D.conjugata* against a panel of common pathogenic bacterial strains.

Organism(s)	MIC mg/ml
Pseudomonas aeruginosa	4.50
Shigellaflexneri	4.50
Escherichia coli	4.50
Salmonella typhi murium	4.50
Salmonella paratyphi	4.50
Salmonella typhi	4.50
Enterobacter sp	9.00
Klebssiella pneumoniae	4.50
Staphylococcus aureus	2.25
Streptococcus mutans	4.50
Staphylococcus citreus	4.50
Staphylococcus epidermidis	4.50

4. DISCUSSION

In the present era, plant and herb resources are abundant, although a large number of studies have been used to obtain a wide variety of purified phyto-chemicals; however, very few screening approaches have been attempted to test the anti-bacterial activities of crude plant materials[11,12]. *D.conjugate* represents a rich natural source of anti-microbial agents. Plants are used medicinally in different countries and are important source(s) of many potent and powerful drugs. A wide range of medicinal plants (or their parts) are used to extract raw drugs and they possess varied medicinal properties[13,14]. In the present investigation, the methanolic plant extract of *D.conjugate* was formulated and studied for its anti-microbial activity against 12 common potentially pathogenic bacteria. The MLE of *D.conjugate* showed excellent activity against S. aureus with the average zone of inhibition of 25 mm. The results of this experiment revealed that the MLE of *D.conjugate* is an effective antimicrobial agent for S. aureus. Toda et al., reported that extracts of fern inhibited and *killed S.aureus, S epidermidis, Salmonalla typhi, S. typhimurium, Shigella flexneri, S. dysenteriae* and *Vibrio spp*. including V. cholerae. In the present study, the MIC values for MLE were determined by micro-dilution method. The MLE of *D.conjugate* had the best MIC values against S. aureus and Enterobacter (2,25, and 9.0 µg/ml, respectively) A similar finding was reported for Bordetella pertussis (Horiuchi et al., 1992). The aqueous extracts of *Pteris vitatta* inhibited cariogenic streptococci, including S. mutans; and activity against other harmful mouth flora has been reported in the patented literature also [15] . A potentially valuable anti-cariogenic effect of ferns was suggested by inhibition of the synthesis of insoluble glucans by S. mutans. Also contains flavonols mainly quercetin, kaempferol, myricetin, and their glycosides. *D.conjugate* has been shown to have a wide range of beneficial physiological and pharmacological effect. The use of ferns is clearly still a long way from clinical application but there are promising leads in the dental context [16]. The concept of being able to exploit an antimicrobial agent which is a new chemical entity found in an abundantly available and renewable source is indeed an important achievement. These effects have been attributed in part to the anti-oxidative and free-radical scavenging activities of the polyphenolic components [17]. The anti-oxidant activities were studied by employing various antioxidant assays such as DPPH and hemolytic activity. Evidence(s) have emerged, however, to suggest that these molecules have the capacity to modulate the physical structure of cell membranes [18].Thus a number of membrane-dependent cellular processes, such as cell signaling and the cell cycle , arachidonic acid metabolism, cell proliferation[19] apoptosis and mitochondrial functionality may be influenced by the interaction of catechins with the cellular phospholipid palisade[20].The hemolytic activity, PPL inhibition, cholesterol degradation (cholesterol oxidase) as well as anti oxidant activity of MLE of fern were studied by employing in vitro assay systems. The MLE of *D.conjugate* showed the best MIC values against S. aureus and Enterobacter sp. (2.25 mg/ml, respectively). There are some contradictions over precisely which bacterial species are inhibited by fern as previously[21] S. typhimurium and Campylobacter jejuni have been reported to be both resistant and susceptible to methanolic extract of *D.conjugate* differences in the observations might be seen because of bacterial strain variation, the sources and infusions/ extracts strengths of various teas used and the definition of being susceptible or resistant. Our study also provided a strong evidence of ability of the MLE of *D.conjugate* to inhibit the activity of mammalian lipase (PPL) in vitro. The MLE showed 55% anti-lipolytic activity against PPL as well as cholesterol oxidase inhibition (69%). The present study thus in deed provided strong experimental evidences that the MLE of *D.conjugate* is not only a potent source of natural anti-oxidants and anti-bacterial activities but also possesses efficient cholesterol degradation and anti-lipolytic potential that might be beneficial in the human body weight management as well as obesity-related diseases.

5. CONCLUSION

The methanolic extract of has the potential to inhibit the growth of many common bacterial pathogens. There are still scanty studies on anti-bacterial properties of *D.conjugate* or their biochemical constituents. It may be suggested that the plants/ tea extracts may possess effective antimicrobial activities that may be explored in the management of common bacterial infectious diseases. The *D.conjugate* may represent new source(s) of antimicrobial phyto-chemicals with stable, biological activity that can extend a scientific base for the use of tea in the modern medicine. The need for screening of and other known or unknown medicinal plants becomes more compelling because of indiscriminate/ irrational use of potent antibiotics, many bacteria/ microorganisms have developed genetic modification to overcome bactericidal/ bacteriostatic effects of commonly used antibiotics. Most of the diseases against which the lycophytes are said to have curative properties, are caused by both Gram-positive and Gram-negative bacteria. The discovery of novel phyto-constituents that are likely to have least toxicity and/ or undesired side-effects is a much desired event. Finally, it could be

concluded that the MLE of *D.conjugate* not only possessed strong to moderate anti-bacterial activities against 12 common pathogenic and/ opportunistic bacteria but also markedly effected the degradation of cholesterol in vitro as well as appeared to be an effective anti-lipolytic preparation. MLE of *D.conjugate* appeared to be a potent source of natural anti-oxidants and anti-bacterial activities besides possessing efficient cholesterol degradation and anti-lipolytic potential that might be beneficial to improve human health. The active principles involved in this plant need to be purified and individually studied for their active antibacterial, anti-lipase and anti-cholesterol oxidase biochemical constituents.

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