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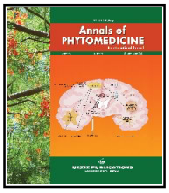

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Phytochemical screening, antioxidant and anti-inflammatory activity of vulnerable tree species, *Diospyros candolleana* Wight

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Abstract

Plant products or their by-products have always exhibited appreciable and vast economic values, as the whole plant or part of it is beneficial for human welfare. One of the significant value is they are treasure of medicines due to the presence of unique kinds of bioactive compounds. Hence, boundless research has been underway on the plants, which leads to the innovation of medicines. To know about the presence of bioactive compounds in the selected tree species, *Diospyros candolleana* (vulnerable and medicinal species) was subjected to screen the preliminary phytochemicals and antioxidants in the leaf, stem, and flower of various solvent extracts. The results were positive for the presence of saponins, tannins, terpenoids, phenols, glycosides, steroids, flavonoids and carbohydrates. Stem ethanolic extract showed the highest percentage of total phenolic content (24.919% at 50 µg/ml). Leaf ethanolic extract (98.78%) showed highest percentage of free radical scavenging activity and reducing power activity was more in stem ethanolic extract with the absorbance 1.5683. Stem ethanolic extract showed maximum 47% of inhibition of protein denaturation and 62% of proteinase inhibitory activity.

1. Introduction

Plants are a boon for all living creatures because one or the other ways they full fill all the necessities for daily life and also they are the major source of medicines. This property is due to the presence of biomolecules and bioactive compounds. Medicinal plants are rich sources of bioactive compounds; currently, the demands of herbal medicine, urge researchers to know the presence of bioactive compounds in medicinal plants due to their effective impact in the medicinal field. That's why the present work on the vulnerable medicinal tree species, *D. candolleana*, belonging to the family Ebenaceae was undertaken.

Diospyros is the largest, pantropical genus of Ebenaceae and it is one of the largest genera in angiosperms having 500-600 species, occurring in the Pacific area and Asia. The species population were found in Africa (94 species), Americas (100 species), Australia (15 species), New Caledonia (31 species), Madagascar and the Comoros (98 species). In this genus, root bark, stem, leaves, fruit and seeds has many medicinal importance, *e.g.,* *D. malabarica* bark is used as astringent, febrifuge, *etc.,* and leaves used in dyspepsia. The timber wood (*e.g. D. ebenum*, *D. japonica*) and fruit (*D. virginiana*) is the most valuable and economically important products of ebony (Wallnöfer, 2001; Halim *et al.,* 2014). *D. candolleana* root bark decoction is used to cure swellings and joint pain (Dev *et al*., 2012) and it is economically important for its timber. On this species research work has been carried out on antimicrobial, preliminary phytochemical analysis, compound characterization, *etc.,* but mainly focused on root, leaf and fruits of *D. candolleana*. Since, no work has been done on antioxidant activity and *in vitro* anti-inflammatory activities, the present study on stem, leaf and flower of *D. candolleana* has been undertaken.

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2. Materials and Methods

2.1 Collection of plant material

Diospyros candolleana Wight leaf, stem, and flower were collected from Agumbe region of Karnataka, India (Figure 1). The plant was authenticated by Dr. K.K. Sampathkumara and the Herbarium (specimen voucher number UOMBOT22DC33) was deposited at Department of Studies in Botany, Manasagangotri, University of Mysore, Mysuru, Karnataka, India.

2.2 Phytochemical screening

The preliminary phytochemical screening was conducted based on the standard procedure of Raaman (2006), Trease and Evans (2009, 1989), and Sofowora (1982, 1993). The leaves, stem and flowers of *D. candolleana* were shade dried and powdered. 30 g was weighed and then subjected to Soxhlet extraction with 450 ml of solvents (based on the polarity, six solvents were used, *viz*., hexane (hex), chloroform (chl.), ethyl acetate (ea.), ethanol (eth.), methanol (met.) and aqueous (Aq.). The extracts were dried and stored in an airtight container for further use.

2.3 Determination of antioxidant activities

2.3.1 Determination of DPPH radical scavenging activity

The leaf, flower, and stem of *D. candolleana* plant extracts were subjected to DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) radical scavenging activity by using the standard protocol of Blois (1958); Gadallah and Ashoush (2016) with ascorbic acid as standard.

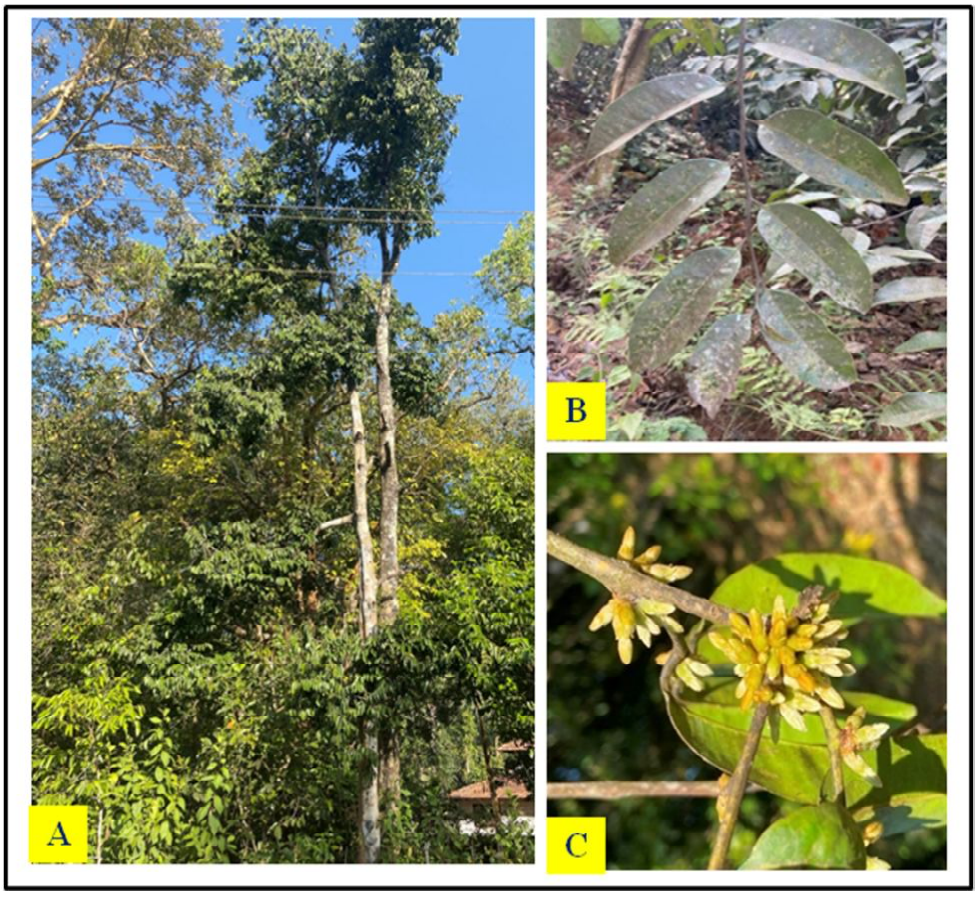

Figure 1: Habit of *Diospyros candolleana* Wight : A. Tree, B. Leaf, C. Flower.

2.3.2 Determination of reducing power assay

The potassium ferricyanide reduction ability (from Fe^{3+} to Fe^{2+}) of plant extract having antioxidant molecules was determined by reducing power assay using the standard method of Oyaizu (1986); Jayanthi and Lalitha (2011), with BHT used as a standard.

2.3.3 Estimation of total phenolic content

The total phenolic content of various plant parts was determined by using the Folin-Ciocalteau method (Singleton *et al*., 1999; Samatha *et al*., 2012).

2.4 *In vitro* anti-inflammatory activity

2.4.1 Protein denaturation assay

The per cent inhibition of protein denaturation assay was conducted by using the procedure of Gunathilake *et al.* (2018).

2.4.2 Proteinase inhibitory activity

To measure the proteinase inhibitory activity, procedure given by Gunathilake *et al.* (2018) was followed.

2.4.3 Membrane stabilization assay

Membrane stabilization assay of the given sample was carried out by following the protocol given by Sakat *et al.* (2010). The percentage of inhibition of haemolysis was determined by following two assays, *viz*., heat induced haemolysis and hypotonicity-induced haemolysis.

2.4.4 Statistical analysis

The experiment was repeated, values represented in Mean ± SE, followed by the same letters within the column are not significantly different at ($p<0.05$) according to Tukey's HSD.

3. Results

3.1 Phytochemical screening

The study of phytochemical screening revealed the presence of promising phytochemical compounds in various extracts of stem, leaf, and flower such as saponins, terpenoids, flavonoids, phenol, glycosides, tannins, steroids, and carbohydrates. Terpenoids were present in all the extracts except leaf chloroform extract and saponins were present in ethanolic, methanolic, and aqueous extracts of all the parts. Flavonoids and tannins were present in ethanolic and methanolic extracts of all the parts and flavonol glycoside was present in ethanolic extracts of all the parts. Phenolic compounds showed positive results only for the methanolic stem, flower, and ethanolic flower extracts. Glycosides

showed positive results for ethanolic, and methanolic extracts of all the parts and only in leaf aqueous extract. Steroids showed positive results for all the flower extracts except the aqueous extract and also present in all parts of ethanolic and methanolic extracts except leaf methanolic extract. Carbohydrates showed a positive result for all the extracts (Table 1).

Table1: Phytochemical screening of *D. candolleana* leaf, stem and flower

Phytochemical tests		Hex			Chl			Ea			Eth			Meth			Aq		
		L	S	F	L	S	F	L	S	F	L	S	F	L	S	F	L	S	F
Saponins		-	-	-	-	-	-	-	-	-	++	++	+	+	++	+	+	+	+
Terpenoids		++	+	+	-	+	+	+	++	+	++	++	++	+	+	+	+	+	+
Flavanoids	1. Conc. HCl	-	-	-	-	-	-	-	-	-	+	++	++	+	+	+	-	-	-
	2. NaOH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3. Magnesium reduction	-	-	-	-	-	-	-	-	-	++	++	++	-	-	-	-	-	-
Phenol	1. $FeCl_3$	-	-	-	-	-	-	-	-	-	-	-	++	-	++	++	-	-	-
Glycosides		-	-	-	-	-	-	-	-	-	++	++	+	+	+	+	+	-	-
Tannins	1. $FeCl_3$	-	-	-	-	-	-	-	-	-	++	++	-	++	-	-	-	-	-
	2. Potassium dichromate	-	-	-	-	-	-	-	-	-	++	++	++	+	++	+	-	-	-
Steroids	1. Lieberman Burchard test	- -	-	+	-	-	+	-	-	+	+	++	++	-	+	++	-	-	-
	2. H_2SO_4	-	-	+	-	-	+	-	-	+	+	++	++	-	+	++	-	-	-
Carbohydrates	1. Fehling's test	+	+	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+
	2. Molish test	+	++	+	+	++	+	+	++	+	+	++	+	+	++	+	+	+	+
	3. Benedict's test	-	-	-	-	-	-	-	-	-	++	++	+	+	+	+	-	-	-
	4. Barfoed's test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: "+" positive with less amount, "++" positive with more amount, "-" negative, Hex: Hexane, Chl: Chloroform, Ea: Ethyl acetate, Eth: Ethanol, Meth: Methanol, Aq: Aqueous "S": stem, "F": flower, "L": leaf.

3.2 Determination of antioxidant activities

Antioxidant activity of *D. candolleana* stem, leaf, and flower extracts showed promising antioxidant activity in DPPH and reducing power assay, and also exhibited higher total phenolic content. Hydroxyl ion and nitric oxide activity were also done for stem, leaf, and flower extracts but showed very less activity in all the extracts.

3.2.1 Estimation of total phenolic content

The total phenolic content of *D. candolleana* stem, leaf, and flower extracts showed the presence of phenolic content by the appearance of light blue color to a deep blue color concerning higher content of phenol and was measured at 725 nm, using gallic acid as standard. Among the stem, leaf, and flower extracts, stem methanolic extract showed the highest percentage of total phenolic content (24.919% at 50 µg/ ml), and stem hexane extract showed very less percentage of total phenolic content (2.4745% at 50 µg/ ml). Among the six solvent extracts, ethanolic and methanolic extracts of stem, leaf, and flower extracts showed the highest. Percentage of total phenolic content and less activity in hexane and chloroform extracts (Figure 2 and Table 2).

Table 2: Total phenolic activity of *D. candolleana* stem, leaf and flower

Extracts	Stem (50 µg/ml)	Leaf (50 µg/ml)	Flower (50 µg/ml)
HEX	02.47 ± 0.04	03.83 ± 0.20	03.79 ± 0.07
CHL	04.74 ± 0.01	04.57 ± 0.03	04.28 ± 0.14
ETH	13.86 ± 0.39	08.06 ± 0.01	07.43 ± 0.05
EOH	24.92 ± 0.32	16.21 ± 0.56	15.34 ± 0.18
MET	14.87 ± 0.22	14.53 ± 0.83	10.80 ± 0.30
AQ	03.90 ± 0.01	14.07 ± 0.05	05.53 ± 0.10

3.2.2 Determination of DPPH radical scavenging activity

The DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) activity of *D. candolleana* stem, leaf, and flower extracts revealed significant results of inhibition ($p \geq 0.05$) in a dose-dependent manner. The decrease in absorbance indicated an increase in free radical scavenging activity concerning the color changes from deep purple to pale yellow color. The results showed highest percentage of activity in ethanolic and methanolic extracts of the stem, leaf, and flower

100 µg/ml. Leaf ethanolic extract (98.78%) and stem ethanolic (95.82%) revealed the highest percentage of free radical scavenging activity with an IC_{50} of 4.4889 µg/ml and 6.0078 µg/ml, respectively, compared to standard ascorbic acid (99.98%, with IC_{50} 3.6539) and the less percentage of radical scavenging activity in leaf hexane 8.79% with IC_{50} of 698.0226 µg/ ml (Figures 3, 4 and Table 3).

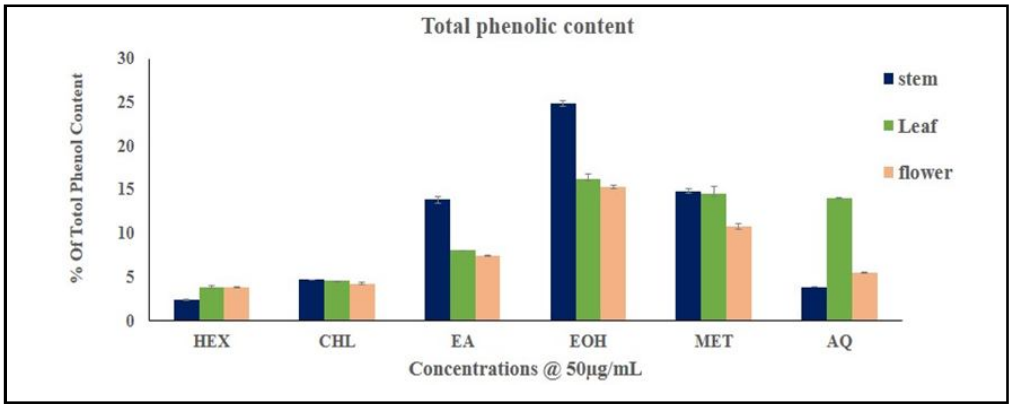

Figure 2: Per cent total phenolic content of *D. candolleana* stem, leaf and flower.

Table 3: Per cent radical scavenging activity and IC_{50} values of *D. candolleana* stem, leaf and flower

Extracts	% RSA (100 µg/ml)			IC_{50} values (µg/ml)		
	Stem	Leaf	Flower	Stem	Leaf	Flower
HX	33.08 ± 0.18	08.79 ± 0.04	09.57 ± 0.06	475.06	698.02	796.75
CHL	14.98 ± 0. 33	39.86 ± 0.89	35.16 ± 0.56	337	133.04	132.53
ETH	93.98 ± 0.10	92.06 ± 0.12	42.58 ± 0.49	28.01	29.69	129.57
EOH	95.82 ± 0.16	98.78 ± 0.10	94.04 ± 0.24	6.01	4.49	13.45
MET	93.64 ± 0.22	95.68 ± 0.70	92.84 ± 0.27	19.17	25.96	26.9
AQ	38.71 ± 0.71	93.11 ± 0.86	29.32 ± 0.82	129.13	24.49	172.79
ASCO		99.98 ± 0.01			3.65	

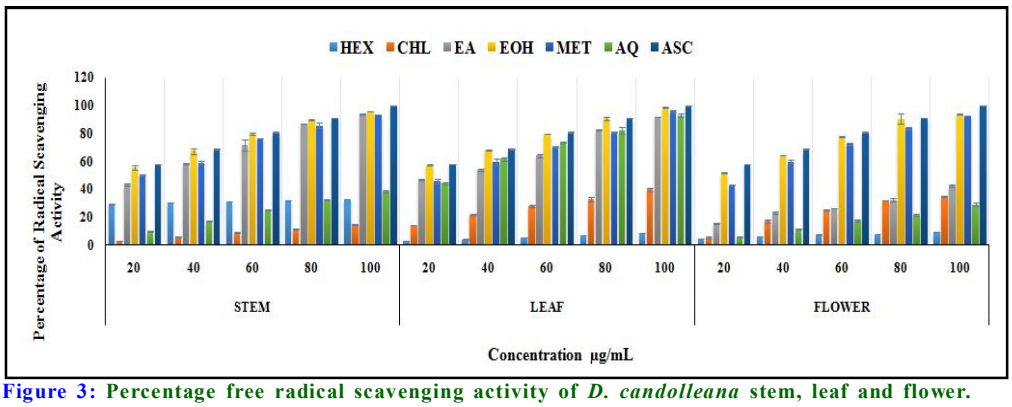

Figure 3: Percentage free radical scavenging activity of *D. candolleana* stem, leaf and flower.

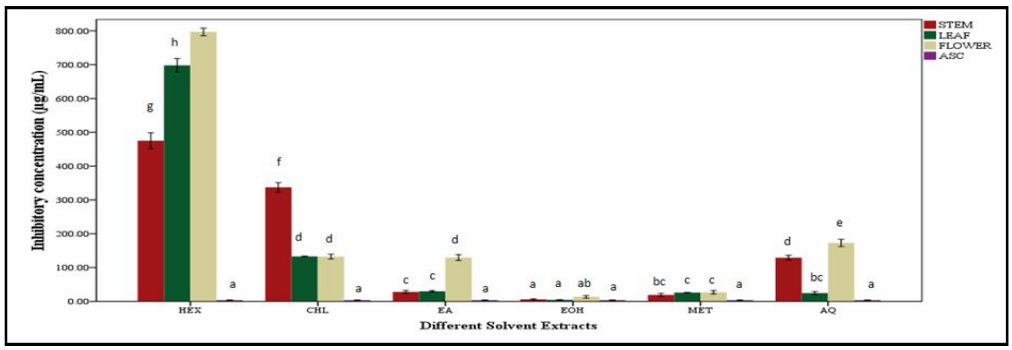

Figure 4: IC_{50} values of radical scavenging activity of *D. candolleana* stem, leaf and flower.

3.2.3. Determination of reducing power assay

The reducing power assay of *D. candolleana* stem, leaf, and flower was measured at 700 nm. An increase in absorbance indicated an increase in reducing activity with the color changes from light green to dark green color. The activity was more in ethanolic extracts of all three extracts, methanolic extracts of leaf, and stem, and also leaf aqueous extract. Stem ethanolic extract showed the highest reducing power activity followed by leaf ethanol, leaf aqueous, stem methanol, flower ethanol, and leaf methanol with the absorbance 1.5683, 1.4207, 1.3743, 1.2393, 1.223, and 1.1557, respectively, at 100 µg/ml compared to standard BHT (1.8777). The least activity was shown by flower hexane extract with an absorbance of 0.0993 at100 µg/ml (Figures 5, 6 and 7) and their corresponding values were presented in the Table 4.

Table 4: Reducing power activity of *D. candolleana* stem, leaf and flower

Extracts	Stem	Leaf	Flower
HX	0.15 ± 0.00	0.14 ± 0.00	0.10 ± 0.00
CHL	0.30 ± 0.02	0.26 ± 0.01	0.17 ± 0.01
ETH	0.90 ± 0.05	0.87 ± 0.00	0.62 ± 0.00
EOH	1.57 ± 0.03	1.42 ± 0.01	1.22 ± 0.02
MET	1.24 ± 0.02	1.16 ± 0.02	0.85 ± 0.02
AQ	0.40 ± 0.01	1.37 ± 0.01	0.21 ± 0.00
BHT		1.88 ± 0.01	

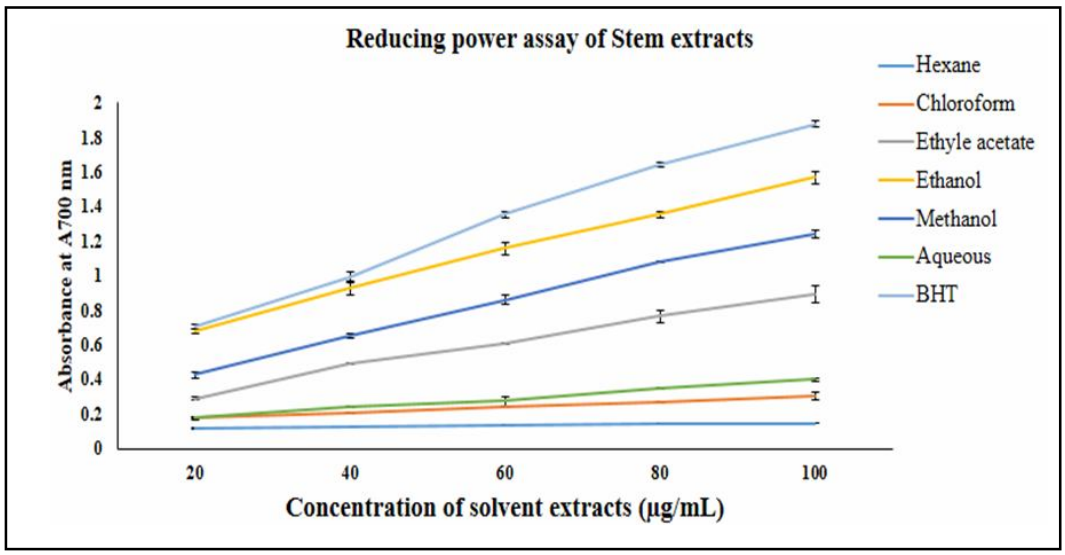

Figure 5: Reducing power activity of various solvent extracts of *D. candolleana* stem.

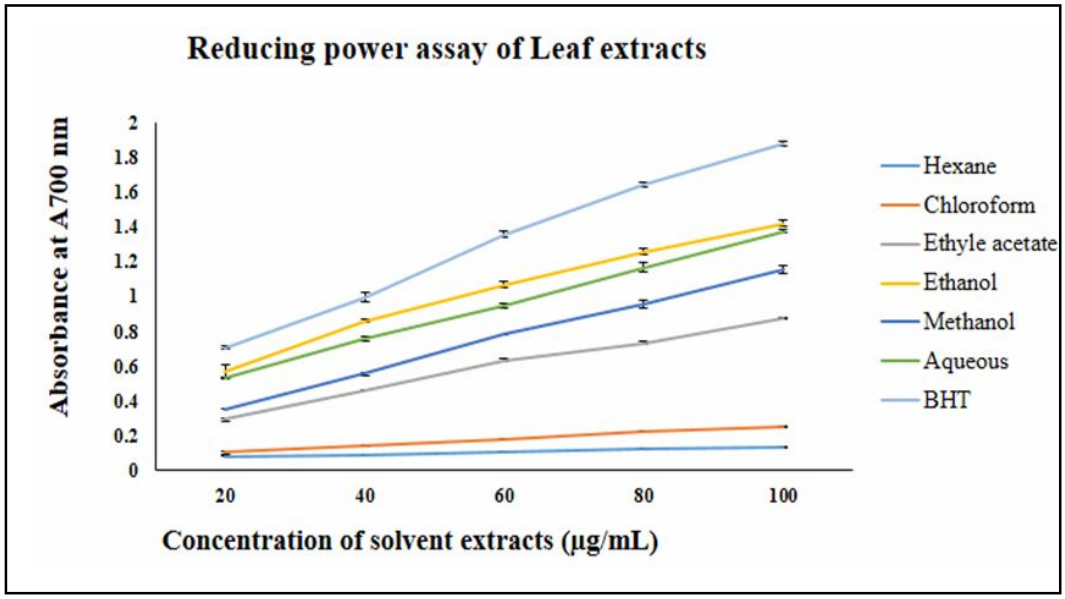

Figure 6: Reducing power activity of various solvent extracts of *D. candolleana* leaf.

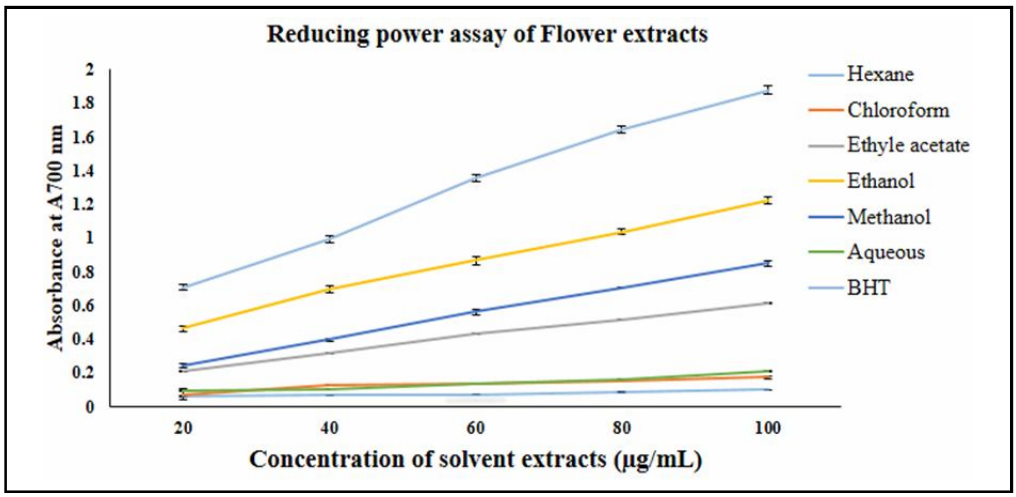

Figure 7: Reducing power activity of various solvent extracts of *D. candolleana* flower.

3.3 *In vitro* anti-inflammatory activity

D. candolleana has a medicinal property in root bark (cure joint pain and swelling), based on this, we have checked whether stem has any anti-inflammatory property, by performing three *in vitro* anti-inflammatory assays. The present study of antioxidant activities revealed that, ethanolic extract has more activity among all other extracts. So, the stem ethanolic extract was chosen for the anti-inflammatory activity.

3.3.1 Protein denaturation assay

Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on the changes in the anti-inflammatory activity, the ability of plant extracts on protein denaturation was studied. The effect of extract at different concentrations on protein denaturation inhibitory activity results showed that the protein denaturation inhibition ability significantly increased ($p<0.05$) with increasing concentration. The maximum percent inhibition of protein denaturation was observed at 3 mg/ml concentration (47%), followed by 29% denaturation at 2 mg/ml concentration. Significantly lower protein denaturation inhibition ability was observed at lower concentration that is 1mg/ml (15%). The results also showed percent inhibition protein denaturation in a dose-dependent manner and the maximum inhibition of denaturation of albumin was found at higher concentration, by decreasing in concentration showed less inhibition of protein denaturation efficacy (Figure 8 and Table 5).

Table 5: Percentage denaturation of protein at different concentration

Treatments	Conc.(mg/ml)	Absorbance at 660 nm	% Protein denaturation
S1	1 mg	0.59 ± 0.59	15
S2	2 mg	0.54 ± 0.54	29
S3	3 mg	0.44 ± 0.44	46
Control	-	0.68 ± 0.00	-
Blank	-	0.00 ± 0.00	-

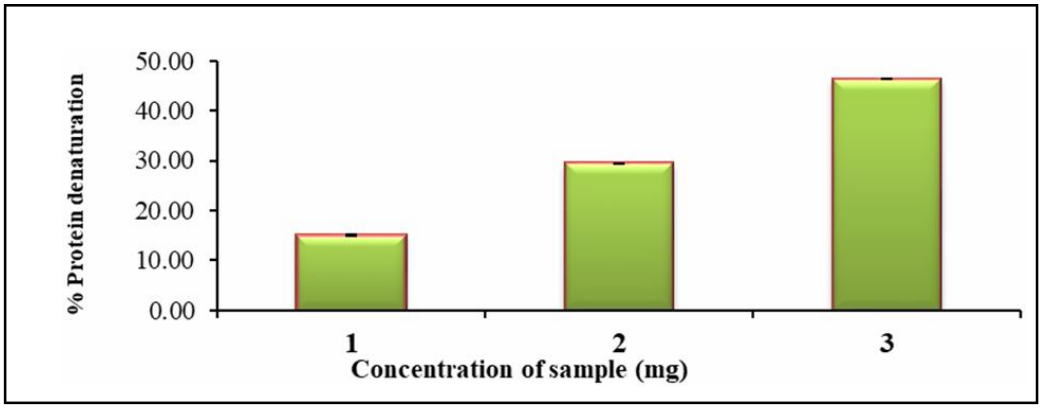

Figure 8: Percentage denaturation of protein at different concentration.

3.3.2 Proteinase inhibitory activity

Earlier report showed that botanicals have efficiency to inhibit protein denaturation. Although, the precise mechanism of this membrane stabilization has yet to be elucidated, it has been proposed that the extract might inhibit the release of the lysosomal content of neutrophils at the site of inflammation. These neutrophils lysosomal constituents include bactericidal enzymes and proteinases, which, upon extracellular release, cause further tissue inflammation and damage. The proteinase inhibition ability of the sample at different concentration is represented in Figure 9 and Table 6.The results significantly showed that lower inhibition percentage at 5 mg concentration (27%), followed by 44% at 10 mg concentration. Maximum percentage inhibition of proteinase was reported at higher concentration 62% at 15 mg concentration.

Table 6: Percentage inhibition of proteinase activity at different concentration

Treatments	Conc. (mg/ml)	Absorbance at 210 nm	% Inhibition of proteinase action
S1	5 mg	0.05 ± 0.02	27
S2	10 mg	0.06 ± 0.03	44
S3	15 mg	0.06 ± 0.04	62
Control	-	0.04 ± 0.00	-

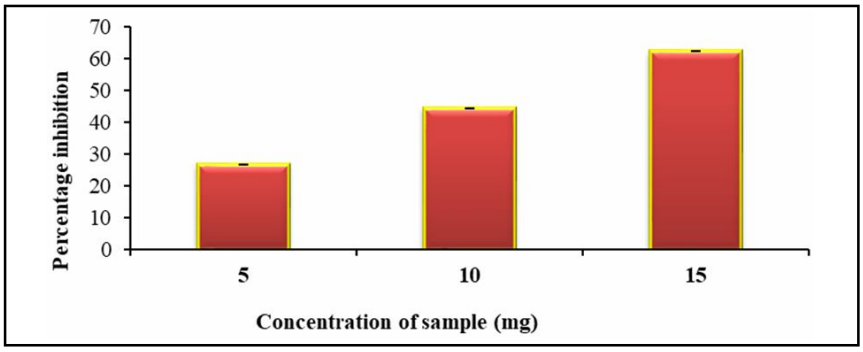

Figure 9: Percentage inhibition of proteinase activity at different concentration.

3.3.3 Membrane stabilization assay

3.3.3.1 Heat induced haemolysis

The samples were tested at the concentrations of 1, 2 and 3 mg/ml. The maximum inhibitory effect was clearly observed at higher level of concentrations and it is within the range of 20% to 55% ($p<0.05$). The maximum percentage of inhibition of 55% was noticed at 3 mg/ml concentrations, followed by 38% at 2 mg/ml and lower inhibition of haemolysis (20%) was recorded at 1 mg/ml concentration and the result is depicted in the Figure 10 and Table 7. Therefore, the obtained results in this study clearly indicated that the higher the concentration of sample leads to maximum inhibition level of haemolysis and decreases the toxicity to erythrocytes.

Table 7: Percentage inhibition of heat induced hemolysis by different concentration

Treatments	Conc.(mg/ml)	Absorbanceat 560 nm	% Inhibition of haemolysis
S1	1 mg	0.22 ± 0.03	20
S2	2 mg	0.20 ± 0.05	38
S3	3 mg	0.19 ± 0.08	55
Control	-	0.23 ± 0.00	

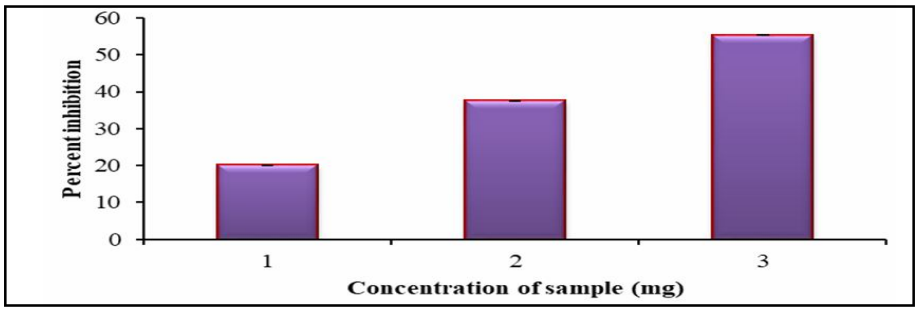

Figure 10: Percentage inhibition of heat induced hemolysis by different concentration.

3.3.3.2. Hypotonicity-induced haemolysis

The results showed that stem ethanolic extract at concentration range of 1-3 mg/ml protect significantly ($p<0.05$) the erythrocyte membrane against lysis induced by hypotonic solution. The sample at 3 mg/ml offered a significant ($p<0.05$) protection against the damaging effect of hypotonic solution. The assay revealed that the sample concentration of 3 mg/ml showed maximum of 46% protection, followed by 26% at 2 mg/ml concentration. Whereas, the sample at 1 mg/ml showed just 11% inhibition of erythrocytic (RBC) haemolysis (Figure 11 and Table 8). The results clearly indicated that the percentage of inhibition of erythrolytic heamolysis increased with increasing concentrations of the samples, hence, it is said to be dose dependent.

Table 8: Percentage inhibition of hypotonicity induced hemolysis by different concentration

Treatments	Conc. (mg/ml)	Absorbance at 560 nm	% Inhibition of haemolysis
S1	1 mg	0.36 ± 0.03	11
S2	2 mg	0.32 ± 0.04	26
S3	3 mg	0.28 ± 0.07	46
Control	-	0.37 ± 0.00	-
Blank		0.01 ± 0.00	-

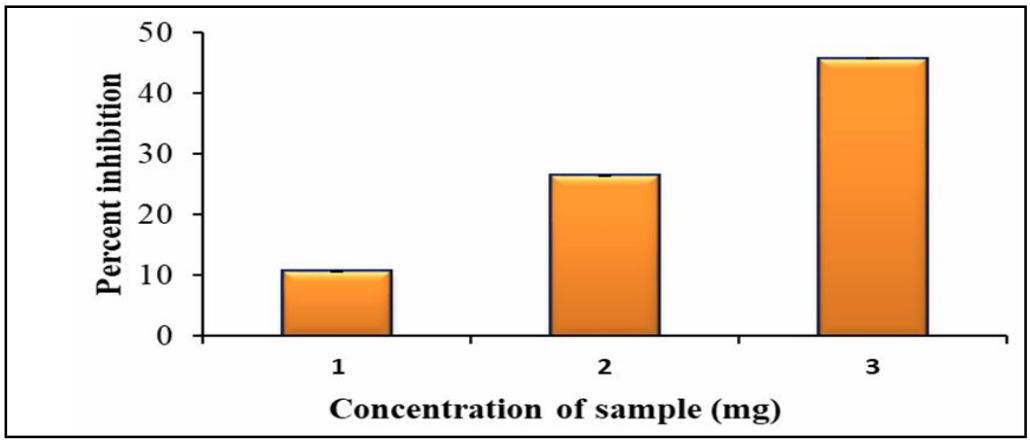

Figure 11: Percentage inhibition of hypotonicity induced hemolysis by different concentration.

4. Discussion

Preliminary phytochemical analysis done for the different solvent extracts of stem, leaf and flower parts of *D. candolleana* showed more phytoconstituents like saponins, terpenoids, flavanoids, phenol, glycosides, tannins, steroids and carbohydrates in ethanolic and methanolic extracts compared to other solvent extracts. Terpenoids and carbohydrates were present in all the extracts while proteins, alkaloids, anthroquinone, phlobotanins, fixed oils and fats were absent in all the extracts. Thomas *et al.* (2013) reported the presence of terpenoids, cardioglycosides, flavanoids, phenols, saponions, tannins, and carbohydrates in *D. chloroxylon*in acetone and methanol extracts. Saponins, anthraquinones, terpenoids, tannins, polyphenols were present in roots, twigs and leaves of *D. lycioides*. Flavanoids and polyphenols and tannins in roots, leaves and twigs but tannins were absent in the roots (Nyambe, 2014). In humans and animals, cholesterol-lowering activity can be done by saponins and also it possess the property of anticancerous, antimicrobial, anti-inflammatory (Aziz *et al.*, 2019), similarly tannins also possess antimicrobial property, phenols, flavonoids and play important role in defence mechanisms. Rashed (2013) reported more radical scavenging activity of Methanolic (89.6%) and ethanolic (70.9%) extracts of *D. Ebenum* while aqueous (47.8%) extract showed less activity. The total phenolic content of *D. melanoxylon* also showed high activity in methanolic (1.78 ± 0.06) and ethanolic (1.36 ± 0.08) extract compared to other solvent extracts, *viz*., acetone (1.16 ± 0.04) and aqueous (1.10 ± 0.10) extracts (Jaiwal *et al*., 2012).

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, which is frequently linked with pain and involves many biological occurrences, such as an increase of vascular permeability, an increase of protein denaturation, and membrane alteration (Ferrero-Millani, *et al.,* 2007). The results obtained from our studies on stem ethanolic extract has shown a potential anti-inflammatory activity. This indicates that *D. candolleana* is more useful in studies of inflammation and in various physiological studies ageing, and diseases such as cancer, neurological disorder, *etc.* The results of anti-inflammatory activity were compared withthe control, which showed significant protection and hemolysis. The extract exhibited membrane stabilization by inhibiting hypotonicity-induced lysis of the erythrocyte membrane and the results were in concordence with the report of Chou (1997). The results of heat induced haemolysis exhibited 55% inhibition at 3 mg/ml concentration. Similar study was conducted by Reshma *et al.* (2014) in plant extracts of *Aegle marmelos* and *Ocimum sanctum* was effective in inhibiting heat-induced haemolysis at 95.64 µg/ml and 42.17 µg/ml, respectively. The proteinase inhibitory action of stem ethanolic extract showed significant action. It was found to be maximum at 15 mg/ml concentration (62%), and the minimum at 5 mg/ml concentration with 27% inhibition.

5. Conclusion

The current study reveals the presence of promising phytochemicals in all solvent extracts of stem, leaf and flower of *D. candolleana*. Ethanolic and methanolic extracts of all parts exhibited more antioxidant properties it indicates the presence of active biochemical compounds. Furthermore, the anti-inflammatory test revealed that the stem ethanolic extract shows moderate anti-inflammatory activity. Therefore, we belive that the research work presented here paves the way for initiating production of high valued biomolecules using biotechnological approaches.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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