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PHARMACOGNOSTICAL OVERVIEW ON *TERMINALIA ARJUNA*: A JUSTIFICATION OF FOLKLORIC BELIEF

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ABSTRACT: The application of medicinal plants such as *Terminalia arjuna* to maintain health and treat disease started since time immemorial and still is a part of medical practice. The phytochemical analysis of bark of *T. arjuna* in methanolic, hydromethanolic (1:1), aqueous and petroleum ether extracts revealed the presence of carbohydrates, amino acid, alkaloids, saponins, tannins, flavonoids, terpenoids, glycosides, xanthoproteins and phenols. *In-vitro* antioxidant capacity by linear regression analysis was measured by assaying DPPH radical and H_2O_2 scavenging capacities. Their respective IC_{50} values were found to be 336.89 µg/ml and 253.01 µg/ml. The hydromethanolic extract gave total phenolic, total flavonoids and FRAP values of 754.16 ± 16.26 mg GAE/g, 247.02 ± 8.81 mg/g QE/g and 1.36 ± 0.03 mM $FeSO_4$ respectively. The IC_{50} values for *in-vitro* anti-inflammatory activities were evaluated by the following assays: lipoxygenase inhibition (IC_{50} = 480.5 µg/ml), heat induced protein denaturation (IC_{50} = 282.32 µg/ml), proteinase inhibition (IC_{50} = 1339.3 µg/ml) and RBC membrane stabilization (IC_{50} = 192.81 µg/ml) at different concentrations using aspirin as control. Studies were also carried out to assess the hypoglycemic potential by assaying the ability of the plant to inhibit alpha amylase (IC_{50} = 210.91 µg/ml) and pancreatic lipase (IC_{50} = 51.52 µg/ml) activities. In addition, glucose binding capacity and glucose diffusion was also monitored). Antimicrobial activity of the extracts was studied against common pathogens. The zone of inhibition was observed using well diffusion method.

INTRODUCTION: In India, over 7500 species of medicinal plants are used by various ethnic communities and play an integral part of their culture [1, 2]. The use of such herbal medicines and nutraceuticals is versatile and has been utilized in various national healthcare settings.

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An estimated 80% of the world's population living in the developing world rely on herbal medicinal products as a primary source of healthcare [3]. One such enthanomedicinally important plant is *Terminalia arjuna*, belonging to the family Combretaceae. Common folkloric uses of the bark include anti-dysentric, antipyretic, astringent, cardiotonic, lithotriptic, anticoagulant, hypo-lipidemic, antimicrobial and antiuremic agent [4].

Studies on the bark of *T. arjuna* have shown high activities of superoxide dismutase, glutathione and catalase levels, demonstrating effectiveness against

oxidative stress [5]. It was found to decrease LDL, HDL and total cholesterol levels thereby lowering blood pressure and frequency of angina-attacks. In addition, it possesses hepatoprotective, anti-mutagenic, antiviral and antibacterial activities [6]. Hence the present study was intended to evaluate the presence of phytoconstituents, pharmaco-gnostical and antimicrobial activity of the bark of *T. arjuna*.

MATERIALS AND METHODS:

Collection and Preparation of Samples: The bark samples of *Terminalia arjuna* were collected from Bengaluru, India in 2016. The bark was cleaned, rinsed in distilled water, sun dried and ground into a fine powder. Crushed samples were extracted using three different solvent systems: distilled water (aqueous), methanol (MeOH) and hydro-methanolic extract (1:1 v/v). Extraction was carried out on an orbital shaker for 24 h at room temperature. Solvents were evaporated under vacuum and the resulting extracts were stored at 4ºC.

Fluorescence Analysis and Phytochemical Screening: Fluorescence characteristics of the powdered seed with different chemicals were observed in daylight and ultraviolet light [7]. Phytochemical examinations were carried out for all the extracts as per the standard methodology to confirm the presence of phytoconstituents [8, 9].

Detection of Carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates as follow:

Molisch's Test: Formation of the violet ring at the junction with 2 drops of alcoholic α-naphthol solution indicates the presence of carbohydrates.

Benedict's Test: Orange red precipitate with Benedict's reagent indicates the presence of reducing sugars.

Fehling's Test: Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A and B solutions. Presence of reducing sugars was indicated by the formation of a red precipitate.

Detection of Alkaloids: Extracts were dissolved individually in dilute HCl and filtered. Tests for the presence of alkaloids were as follows:

Mayer's Test: Formation of a yellow coloured precipitate with Mayer's reagent indicates the presence of alkaloids.

Wagner's Test: Formation of brown / reddish precipitate with Wagner's reagent indicates the presence of alkaloids.

Dragendroff's Test: Formation of red precipitate with Dragendroff's reagent indicates the presence of alkaloids.

Detection of Saponins:

Foam Test: Persistence of foam after 10 min with 0.5 ml extract and 2 ml water, shaken well indicates the presence of saponins.

Detection of Glycosides: Extracts were hydrolyzed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test: Extracts were treated with $FeCl_3$ solution and immersed in boiling water for about 5 min. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammoniacal layer indicates the presence of anthranol glycosides.

Detection of Phytosterols:

Salkowski's Test: The plant extracts were treated with $CHCl_3$ and filtered. The filtrate thus obtained was mixed with few drops of concentrated H_2SO_4, shaken and allowed to stand. The appearance of golden yellow colour was indicative of triterpenes.

Detection of Flavonoids:

Lead Acetate Test: Formation of yellow colour precipitate with the extract and a few drops of lead acetate solution indicates the presence of flavonoids.

Detection of Proteins and Amino Acids:

Xanthoproteic Test: Formation of yellow colour with extract and concentrated HNO_3 indicates the presence of proteins.

Ninhydrin Test: Formation of blue colour with extract and 0.25% w/v ninhydrin reagent boiled for a few minutes indicates the presence of amino acid.

Millon's Test: A reddish-brown coloration with the extract and Millon's reagent gently heated indicates the presence of tyrosine residue

Detection of Tannins:
Ferric Chloride Test: Formation of bluish-black colour with the extract and 3 - 4 drops of $FeCl_3$ indicates the presence of phenols.

Detection of Terpenoids:
Salkowski Test: Appearance of reddish brown colour with 5 ml of extract, few drops of $CHCl_3$ and 3 ml concentrated H_2SO_4 revealed the presence of terpenoids.

Total Phenolic, Flavonoid and FRAP Activity: Total phenolic contents were estimated according to the spectrophotometric method using gallic acid as standard [10] and expressed in terms of gallic acid equivalent (mg of GAE/g of tissue). Aluminum chloride colorimetric method was used for determination of total flavonoids [11] and expressed in terms of quercetin equivalent (mg of QE/g of tissue). The ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) method was used to measure the reducing capacity of plant extracts [12].

Evaluation of Antioxidant Activity using various *in-vitro* Methods:
DPPH Radical Scavenging Activity: Standard ascorbic acid was pipetted out into different test tubes (100 - 500 µg/ml). 0.1 ml solution of each dilution was taken and made up to 3 ml with DPPH (20 µg/ml). The test tubes were incubated for 10 min at room temperature. The contents of each tube were mixed well, and the absorbance was measured at 517 nm against a blank [13]. The percentage inhibition of DPPH by the samples was calculated as follows:

% Inhibition = (OD of control – OD of sample) × 100 / OD of control .....(1)

Hydrogen Peroxide Scavenging Activity: Hydromethanolic extract of *T. arjuna* in 3.4 ml phosphate buffer was added to 0.6 ml of 45 mM H_2O_2 solution and the absorbance of the reaction mixture was recorded at 230 nm against a blank containing only buffer [14]. Ascorbic acid was used as standard. The concentration of H_2O_2 (mM) in the assay medium was determined using a standard curve ($y = 0.1223x + 29.62$; $R^2 = 0.586$). H_2O_2 scavenging ability was calculated as IC_{50}. The percentage inhibition was calculated as in Eq. 1.

Evaluation of Anti-inflammatory Activity using various *in-vitro* Methods:
Lipoxygenase Inhibition: Soybean lipoxygenase activity was assayed [15]. The reaction contained 2.9 ml 0.1M borate buffer pH 9.0 and 50 µl 10 mM linoleic acid. The reaction was initiated by the addition of 50 µl of the soybean enzyme extract. The enzyme activity was measured by following the formation of the product, 12-HETE at 234 nm for up to 1 min. The enzyme inhibition was determined by pre-incubating the enzyme with the hydromethanolic extract prior to determining its 12-LOX activity. The percentage inhibition was calculated as in Eq. 1. IC_{50} was calculated from $y=0.0839x+8.61$; $R^2=0.788$.

Proteinase Inhibitory Action: The test was performed according to the modified method of Sakat [16]. 2 ml of the reaction mixture contains 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was incubated at 37ºC for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in duplicate. The percentage inhibition of protein denaturation was calculated using Eq. 1. IC_{50} was calculated from $y=0.0399x+4.428$, $R^2=0.8585$.

RBC Membrane Stabilization Activity: Various concentration of hydromethanolic extract, reference sample and control were separately mixed with 1 ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of hRBC suspension. Diclofenac sodium (100µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37 ºC for 30 min and centrifuged at 3000 rpm. The supernatant was used to estimate the haemoglobin content using a spectrophotometer at 560 nm [17]. The percentage of hemolysis was estimated assuming that the control

produced 100% haemolysis. The percentage inhibition of protein denaturation was calculated using Eq. 1. IC_{50} was calculated from $y=0.1525x+39.78$, $R^2= 0.5502$.

Evaluation of Hypoglycemic Activity using various *in vitro* Methods:

Alpha Amylase Inhibition Assay: The procedure described by Shai *et al*., [18] with slight modifications was used to determine the α-amylase inhibitory activity of the fractions. A volume of 250 µl of each hydromethanolic extract fraction or acarbose at different concentrations (100 - 500 µg/ml) was incubated with 500 µl of porcine pancreatic amylase (2 U/ml) in phosphate buffer (100 mM, pH 6.8) at 37 °C for 20 min. Thereafter, 250 µl of 1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was further added to the reaction mixture and incubated at 37 °C for 1 h. Dinitrosalicylate colour reagent (1 ml) was then added and boiled for 10 min. The absorbance of the resulting mixture was read at 540 nm the inhibitory activities of the fractions on *α*-amylases were calculated by using the following formula:

$$\% \text{ Inhibition} = 1 - \frac{B\text{-}b}{A\text{-}\alpha} \times 100$$

Where 'A' is the activity of the enzyme without inhibitor, 'a' is the negative control without the inhibitor, 'B' is the activity of the enzyme with inhibitor, and 'b' is the negative control with inhibitor.

Pancreatic Lipase (PPL) Inhibition Assay: This was performed as described by Bustanji *et al*.,[19] with minor modification. The enzyme solution was prepared immediately before use, by suspending crude porcine pancreatic lipase powder in 0.1 M phosphate buffer (pH 7.6) (100 U/ml). The solution was then centrifuged at 2000 rpm for 10 min and the clear supernatant was recovered. Triolein (1% v/v) was used as the substrate for PLL. The hydromethanolic extract of *T. arjuna* (5, 12.5, 25, 100, 125, 250, 500 µg/ml) was preincubated with 200 µl of PPL solution for 5 min at 37 °C, before the addition of 800 µl triolein substrate solution. The absorbance was measured at 450 nm against blank using denatured enzyme in an ELISA reader. The denatured enzyme was prepared by boiling the enzyme solution for 5 min. Orlistat was used as a reference drug.

The extract was dissolved in DMSO at a final concentration not exceeding 1% (v/v). The activity of the negative control was checked in the presence and absence of the inhibitor. The % inhibition was calculated according to the formula (2).

Determination of Glucose Adsorption Capacity: Glucose adsorption capacity of the samples was determined by the method of Ou *et al*.,[20]. The hydromethanolic extract of *T. arjuna* bark (1%) was added to 25 ml of glucose solution of increasing concentration (5, 10, 20, 50, and 100 mmol/l). The mixture was stirred well, incubated in a shaker at 37 °C for 6 h, centrifuged at 5000 rpm for 20 min and the glucose content in the supernatant was determined. Metronidazole was used as control. The concentration of bound glucose was calculated using the following formula:

Glucose bound = [Initial Glucose] – [Glucose after 6h] × Volume of Solution / Weight of Sample

***In-vitro* Glucose Diffusion Inhibitory Assay:** To study *in-vitro* effects of the hydromethanolic extract of *T. arjuna* bark, on the movement of glucose, a simple model system was adapted as mentioned by Ahmed *et al*., [21] This model involved the use of dialysis tubes that were sealed containing 25 ml of 20 mM glucose and 1% *T. arjuna* bark extract against 200 ml of distilled water at 37 °C in a shaker water bath. The amount of glucose in the external solution was measuredat 30, 60, 120, and 180 min. Metronidazole was used as the control. Glucose dialysis retardation index (GDRI) was calculated using the following formula:

GDRI% = Glucose content with addition of sample × 100 / Glucose content of control

Screening for Antibacterial Activity of Methanolic Extract:

Agar Well Diffusion Assay: The hydromethanolic, hot and cold aqueous *T. arjuna* bark extracts were used for evaluation of the antimicrobial activity by the agar well diffusion method [22]. Pure isolates of each microorganism (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) was subcultured on the agar media plates at 37 ºC for 24 h. One plate of each microorganism was taken and a minimum of four colonies were touched with a sterile loop and transferred into normal saline (0.85%) under

aseptic conditions. Density of each microbial suspension was adjusted equal to that of 10^6 cfu/ml (standardized by 0.5 McFarland standards) and used as the inoculum for performing agar well diffusion assay. 100 µl of inoculum of each test organism ± standard deviation were calculated was spread onto the agar plates so as to achieve a confluent growth.

A well of 6 mm diameter was made using a sterile cork borer. 100 µl volume of each extract was placed directly into the wells (in triplicates) of the inoculated agar plates for each test organism, allowed to stand for 1 h for diffusion of extract into the agar and then incubated at 37 ºC for 24 h. Ciprofloxacin (5 µg/disc) was used as a positive control for antibacterial activity. After 24 h of incubation at 37 ºC, zone of inhibition (ZOI) was observed and diameter measured. The experiments were performed in triplicates and the mean values of the diameter of inhibition zones with

Statistical Analysis: Data of *in-vitro* assays recorded were analyzed using Microsoft Excel to determine IC_{50}. One-way analysis of variance (ANOVA) were conducted and $P<0.05$ was considered significant.

RESULTS AND DISCUSSION: Ethnomedicinal plants have been documented in a number of ancient texts for their ability to treat various common ailments as they are a rich source of bioactive molecules. The utilization of these substances as phytochemical agents in the treatment of inflammation has picked up significance all through the world. In the present research work hydromethanolic extract of *T. arjuna* was evaluated for the antimicrobial, antioxidant, anti-inflammatory and antidiabetic properties.

Fluorescence and Phytochemical Analysis: The bark of *T. arjuna* produced different colours and fluorescence under UV light and day light when treated with various reagents **Table 1**.

TABLE 1: FLUORESCENCE ANALYSIS OF POWDERED BARK OF *T. ARJUNA*

S. no.	Experiment	Visible / Day light	UV light (365 nm)
1	Powder as such	Light green	Light green
2	Powder + 1N HCl	Yellow orange	Reddish green
3	Powder + Conc.H_2SO_4	Black	Black
4	Powder + 50% H_2SO_4	Off white	Pink
5	Powder + Conc. HNO_3	Brown	Fluorescent green
6	Powder + Conc. HCl	Brownish red	Black
7	Powder +50% HNO_3	Yellow	Dark green
8	Powder + Acetic acid	Yellowish brown	Light pink
9	Powder + Ferric chloride	Light brown	Brownish green
10	Powder + HNO_3 + NH_3	Yellowish brown	Green
11	Powder + NH_3	Brown	Dark green
12	Powder + Benzene	Colourless	Pinkish red
13	Powder + Petroleum ether	Colourless	Pinkish red
14	Powder + Acetone	Green	Fluorescence orange
15	Powder + Chloroform	Colourless	Pinkish red
16	Powder + Methanol	Brown	Dark green
17	Powder + Ethanol	Light green	Fluorescent pink

TABLE 2: PHYTOCHEMICAL INVESTIGATION OF *T. ARJUNA*

S. no.	Phytochemical analyzed	Test performed	Result			
			MeOH	MeOH : water (1:1)	Aqueous	Petroleum ether
1	Alkaloid	Mayer's	+	++	+	-
		Wagner's	+	+	+	-
		Dragendoroff's test	+	+	-	-
2	Carbohydrate	Molish's	+	+	+	-
		Benedict's	+	+	+	-
		Fehling's	+	+	+	-
3	Saponin	Foam	+	++	+	-
4	Glycosides	Borntrager's	+	+	+	+
5	Steroid	Salkowaski	-	++	+	-
6	Flavonoid	Lead acetate	+	++	+	-
7	Proteins and amino acids	Ninhydrin	+	+	+	-
		Xanthoproteic test	+	+	-	-
		Millon's	+	+	-	-
8	Tannins	Ferric chloride test	+	+	+	-
9	Terpenoids	Salkowski test	+	++	+	-

- indicates absence, + denotes average, ++ means abundance of phytochemicals.

Phytochemicals are secondary metabolites produced by many plants possessing medicinal uses. The analysis of the phytochemical results revealed greater presence of saponins, flavonoids, terpenoids, coumarins, quinines, cardiac glycosides, xanthoproteins, steroids, phenols, carboxylic acid group alkaloids, tannins, terpenoids, glycosides, resins in hydromethanolic extract of *T. arjuna* **Table 2**.

Total Phenolic, Flavonoid and FRAP Activity: Phenolics are known to balance carbohydrate and lipid metabolism, weaken hyperglycemia, dyslipidemia and insulin resistance, invigorate insulin discharge, enhance fat tissue digestion and ease oxidative stress. In addition, they possess physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects [23, 24, 25]. The total phenolic content calculated using the standard curve of gallic acid (y = 0.0096x, R^2 = 0.997) was found to be 754.16 ± 16.26 mg GAE/g of fresh weight tissue respectively **Table 3**.

Flavonoids, the most common secondary metabolite provides health benefits through their antioxidant activity. Their levels as estimated spectrophotometrically using a standard curve of quercetin (y = 0.0014x + 0.0267, R^2 = 0.9988) was found to be 247.02 ± 8.81mg/g fresh weight tissue **Table 3**. The antioxidant potentials of the extracts of the *T. arjuna* bark was estimated from their ability to reduce ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex to a coloured ferrous tripyridyltriazine (Fe^{2+} -TPTZ). The results showed that FRAP values were higher in methanol: water extracts compared to the standard *i.e.*, ascorbic acid **Table 3**. The antioxidant activity of the plant extract is dependent on storage time, geographic origin, harvesting, temperature and light during the storage and solvent used [26].

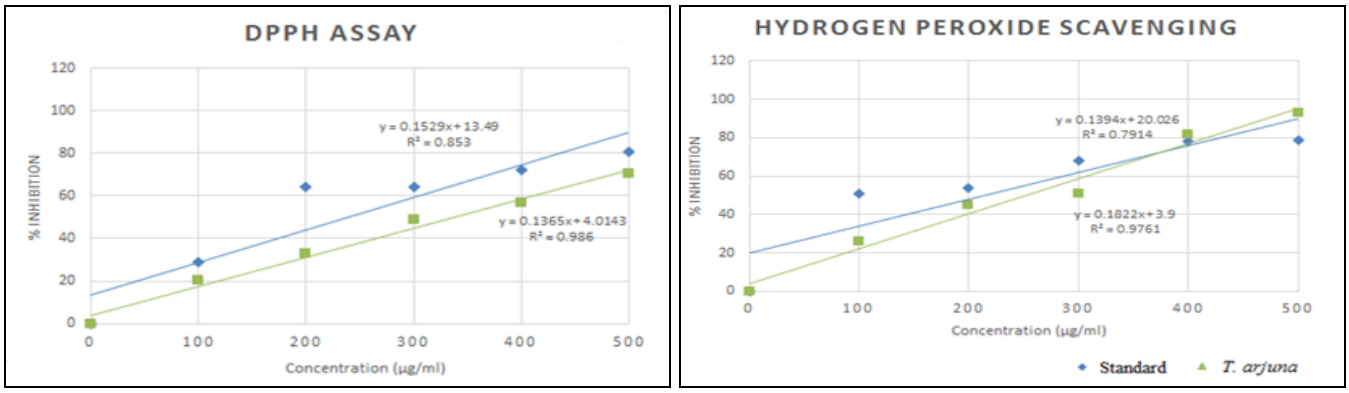
TABLE 3: QUANTITATIVE ANALYSIS OF TOTAL PHENOLS, TOTAL FLAVONOIDS AND FRAP ACTIVITY OF *T. ARJUNA* BARK

Total Phenols (mg GAE/g Fwt)	Total Flavonoids (mg QE/g Fwt)	FRAP activity (mM FeSO4)
754.16 ±16.26	247.02 ± 8.81	1.362 ± 0.03

All values are represented as mean ± SD (n=3). GAE - Gallic acid equivalents; QE - Quercetin equivalents.

***In-vitro* Antioxidant Activity:**

DPPH Radical Scavenging Activity: The total antioxidant capacity of the extracts was calculated using % inhibition against concentration of ascorbic acid (y = 0.1529x + 13.49; R^2 = 0.853) **Fig 1A**. At 500 µg/ml concentration, *T. arjuna* extract exhibited maximum DPPH radical scavenging activity that was found to be 70.3±3.3 %. The IC_{50} values calculated from graph and were found to be 238.78 µg/ml (Ascorbic acid, y = 0.1529x + 13.49), and 336.89 µg/ml (*T. arjuna*, y = 0.1365x + 4.014, R^2 = 0.986) **Table 4**. The results of the present investigation demonstrate that *T. arjuna* can significantly decrease *in-vitro* DPPH radical levels. The antioxidative activities observed is attributed to mechanisms exhibited by different polyphenolic compounds such as tocopherols, flavonoids and other organic acids [27]. Due to their redox properties, they serve as highly effective free radical scavengers causing the quenching of singlet and triplet oxygen or decomposing peroxides.



A B

FIG. 1: ANTIOXIDANT ACTIVITIES OF HYDROMETHANOLIC EXTRACT OF BARK OF *T. ARJUNA* AND ASCORBIC ACID; DPPH RADICAL SCAVENGING (A) AND H_2O_2 SCAVENGING ACTIVITIES (B)

H_2O_2 Scavenging Activity: H_2O_2 scavenging assay was used to monitor the decrease in the absorbance of H_2O_2 upon its oxidation. The H_2O_2 scavenging activity of *T. arjuna* was determined and compared with ascorbic acid **Table 4, Fig. 1B**. The IC_{50} values are calculated from graph and were found to be 215.68 µg/ml (Ascorbic acid, y = 0.1394x + 20.0; R^2 = 0.7914) and 253.01 µg/ml (*T. arjuna*, y = 0.1822x + 3.9, R^2 = 0.9761).

H_2O_2 crosses cell membranes rapidly where it reacts with Fe^{2+} and Cu^{2+} ions to form hydroxyl radicals resulting in various toxic effects. H_2O_2 is a weak oxidizing agent causing enzymatic inactivation by oxidation of essential thiol (-SH) groups. It degrades hemeproteins, such as hemoglobin, to release Fe ions. The ability of the extract to scavenge H_2O_2 is indicative of high antioxidant potential.

TABLE 4: EVALUATION OF *IN-VITRO* ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF HYDROMETHANOLIC *T. ARJUNA* BARK EXTRACTS

	Assay	Control			*T. arjuna* bark	
		Conc. (µg/ml)	% inhibition	IC_{50} (µg/ml)	% inhibition	IC_{50} (µg/ml)
In vitro antioxidant activity	DPPH scavenging activity	100	28.6 ± 9.2		20.2 ± 1.3	
		200	64.35 ± 2.1		32.6 ± 2.4	
		300	64.20 ± 9.8	238.78	48.9 ± 5.6	336.89
		400	72.34 ± 2.9		56.9 ± 8.1	
		500	80.83 ± 3.4		70.3 ± 3.3	
	H_2O_2 scavenging activity	100	51.01 ± 3.4		26.0 ± 0.7	
		200	53.68 ± 2.8		45.2 ± 2.8	
		300	67.93 ± 9.9	215.68	50.8 ± 4.3	253.01
		400	78.21 ± 7.4		81.7 ± 5.6	
		500	78.42 ± 5.6		93.0 ± 0.7	
	LOX inhibiting activity	100	45.14 ± 7.8		24.17 ± 9.6	
		200	48.26 ± 8.3		34.11 ± 5.9	
		300	51.15 ± 10.2	315.5	36.96 ± 9.0	480.5
		400	56.97 ± 11.4		40.65 ± 10.1	
		500	60.3 ± 3.3		48.2 ± 9.4	
In vitro anti-inflammatory activity	Inhibition of heat induced protein denaturation	100	39.1 ± 4.6		32.1 ± 1.03	
		200	51.2 ± 3.8		46.4 ± 0.01	
		300	64.2 ± 10.1	222.85	53.6 ± 2.36	282.32
		400	81.3 ± 7.5		69.2 ± 5.09	
		500	92.1 ± 6.2		72.1 ± 1.03	
	Inhibition of proteinase activity	100	3.15 ± 0.3		5.73 ± 0.6	
		200	6.01 ± 1.3		5.88 ± 0.2	
		300	10.7 ± 2.7	899.1	7.33 ± 2.6	1339.3
		400	21.3 ± 4.8		14.56 ± 5.1	
		500	29.9 ± 5.9		20.72 ± 4.8	
	RBC membrane stabilization	100	62.21 ± 11.2		42.30 ± 7.4	
		200	85.26 ± 8.9		64.11 ± 5.7	
		300	90.67 ± 9.6	121.16	75.78 ±11.2	192.81
		400	95.44 ± 12.1		86.72 ± 9.0	
		500	99.36 ± 6.7		88.9 ± 6.3	

Results are mean ± SEM ($P < 0.05$), obtained from three replicates

***In-vitro* Anti-inflammatory Activity:** Inflammation is a complex physiopathological response to different stimuli. The inflammatory process involves the activity of inflammatory mediators such as neutrophil derived free radical, reactive oxygen species (ROS), nitric oxide (NO), prostaglandins and cytokines. Over production of these leads to tissue injury by damaging macromolecules, lipid peroxidation of membrane and tissue damage play important role in pathogenesis of many inflammatory diseases [28]. Thus, free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation.

Lipooxygenase Inhibition: Lipoxygenases include non-heme iron-containing dioxygenases that involved in the biosynthesis of leukotrienes, needed for the pathophysiology of several inflammatory and allergic diseases [29]. The flavonoid quercetin, abundant in plants behaves as an antioxidant due to

its lipoxygenase-inhibitory activities and free radical-scavenging properties [30]. It does so by binding covalently to iron or by forming molecular complexes blocking access to iron. In general, antioxidants inhibit lipid hydroperoxide formation by scavenging lipidoxy or lipid peroxy- radical formed in course of enzyme peroxidation, thus limiting the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX. The plant lipoxygenase pathway is in many respects equivalent to the 'arachidonic acid cascades' in animals. Hence, *in-vitro* inhibition of lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential. Methanolic extract of *T. arjuna* was assayed at 100 - 500 µg/ml, and the inhibition obtained at different concentrations is as shown in **Table 4.** The standard diclofenac exhibited 60.3 ± 3.3% inhibitions at a concentration of 500 µg/ml. The IC_{50} values are calculated from graph and were found to be 315.5 µg/ml (Diclofenac, y= 0.0971x + 19.36, R^2 = 0.6764), and 480.5 µg/ml (*T. arjuna*, y = 0.0838x + 9.73; R^2 = 0.8528) **Fig. 2A**.

Inhibition of Heat Induced Protein Denaturation: Protein denaturation refers to the loss of protein secondary and tertiary structure by the application of external stress or compounds, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. One cause of inflammation is the denaturation of proteins leading to the loss their biological function. As part of the investigation on the mechanism of the anti-inflammation activity, ability of *T. arjuna* bark extract to inhibit protein denaturation was studied. Maximum inhibition of 72.1% was observed at 500 µg/ml by *T. arjuna*. Diclofenac, a standard anti-inflammatory drug showed the maximum inhibition 92.1% at the concentration of 500 µg/ml compared with control **Table 4**. The IC_{50} values are calculated from graph and were found to be 222.85 µg/ml (Diclofenac, y=0.1715x+11.78, R^2 = 0.945) and 282.32 µg/ml (*T. arjuna*, y = 0.1369x + 11.35, R^2 = 0.914) **Fig 2B**.

Proteinase Inhibitory Activity: Activated leukocytes are widely implicated in cardiovascular disease (CVD). Mononuclear cells are recruited to sites of vascular injury thus contributing to foam cells within atherosclerotic plaques [31]. Leukocyte activation occurs in all the conditions associated with an increased CVD risk: infection, hypertension, hyperlipidemia, hyperglycemia, obesity, and atherosclerosis [32]. Activated WBCs discharge into the surrounding milieu reactive oxygen species (ROS) and a variety of proteolytic enzymes, particularly serine proteases. Proteinases have been implicated in arthritic reactions while leukocytes proteinase plays an important role in the development of tissue damage during inflammatory reactions. Proteinase inhibitors were found to provide protection against such inflammation-induced damage [33]. *T. arjuna* extract and diclofenac exhibited maximum inhibition of 29.9% at 500µg/ml while diclofenac showed a maximum inhibition 20.7% and 29.9% at 500 µg/ml respectively **Table 4**. The IC_{50} values calculated from the graph were found to be 899.1µg/ml (Diclofenac, y = 0.0595x + 3.054, R^2 = 0.937) and 1339.3 µg/ml (*T. arjuna*, y = 0.0376x + 0.359, R^2 = 0.907) respectively **Fig. 2C**.

RBC Membrane Stabilization Activity: The erythrocyte plasma membrane resembles the lysosomal membrane and hence the stabilizing effect of drugs on erythrocyte membrane may correlate with its lysosomal membrane stabilizing effect [32]. Any factor contributing to the lysosomal membrane stabilization leads to the inhibition of release of the inflammatory mediators and consequent inhibition of the process of inflammation. The mechanism of inflammation injury is attributed to the release of ROS from activated neutrophil and macrophages causing tissue injury through lipid peroxidation of membranes. In addition, ROS propagate inflammation by stimulating the release of the cytokines such as Il-I, TNFα, and INFγ, which further stimulate recruitment of additional neutrophil and macrophages. Thus, free radicals are important mediators that provoke or sustain inflammatory processes and consequently their neutralization by antioxidants and radical scavengers can attenuate inflammation. Maximum inhibition was observed at 500 µg/ml, where Diclofenac and *T. arjuna* exhibited 99.36 and 88.98% inhibition respectively **Table 4**. The IC_{50} values for the standard drug and *T. arjuna* was found to be 121.16 (y = 0.172x + 29.16, R^2 = 0.728) and 192.81 (y = 0.1685x + 17.515, R^2 = 0.8671) µg/ml respectively **Fig. 2D**.

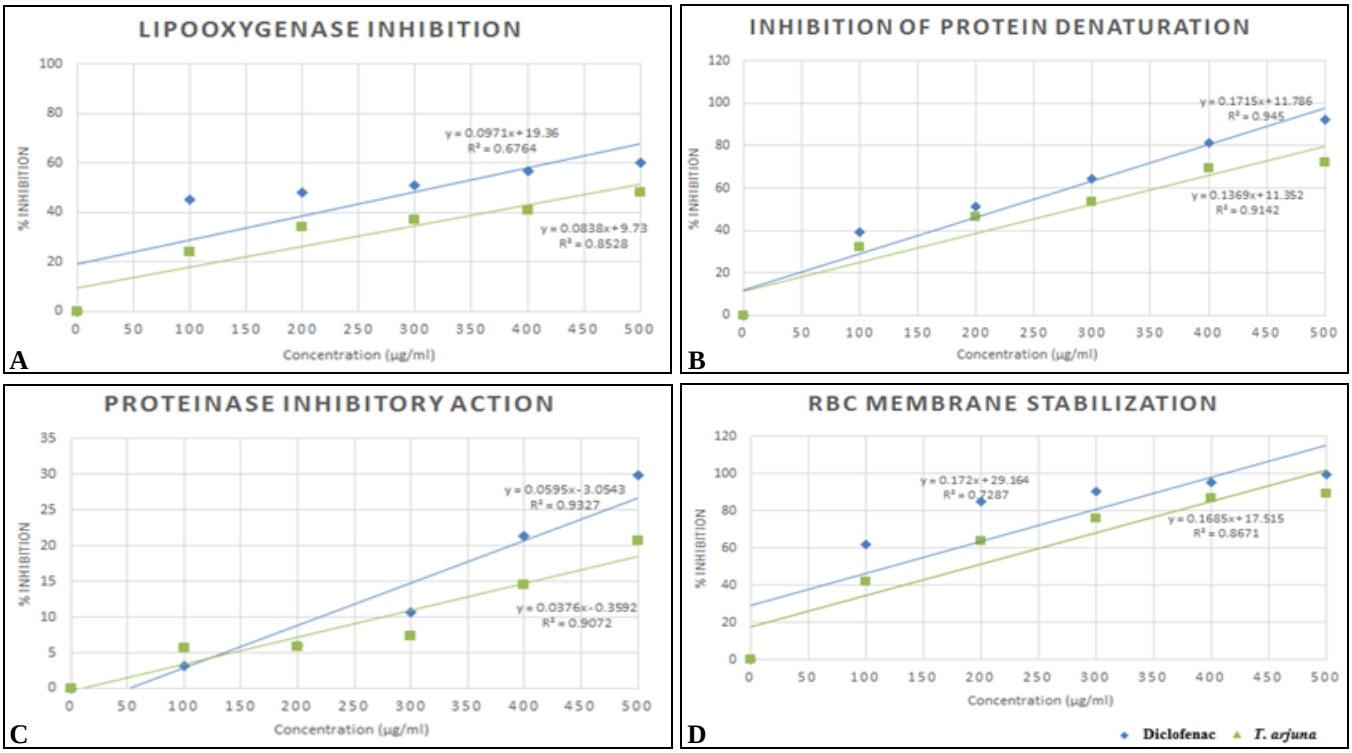


FIG. 2: ANTI-INFLAMMATORY ACTIVITIES OF STANDARD DRUG DICLOFENAC AND HYDRO-METHANOLIC EXTRACT OF *T. ARJUNA* BARK; LIPOXYGENASE INHIBITION ASSAY (A), INHIBITION OF HEAT INDUCED PROTEIN DENATURATION (B), PROTEINASE INHIBITORY ACTIVITY (C) AND RBC MEMBRANE STABILIZATION ASSAY (D)

***In-vitro* Hypoglycemic Activity:** Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia and alterations in carbohydrate, lipid, and protein metabolism, associated with absolute or relative deficiencies in insulin secretion and/or insulin action [31]. The World Health Organization (WHO) has estimated that major burden of diabetes will occur in developing countries, and by 2025, there will be 42% increase (from 51 to 72 million) in the developed countries and 170% increase (from 84 to 228 million) in the developing countries [34]. It is estimated that by 2025, India, China, and the United States will be the top three countries to have large number of diabetics [35]. The high prevalence of diabetes as well as its long-term complications has led to an ongoing search for hypoglycaemic agents. Over the years, various medicinal plants and their extracts have been reported to be effective in the treatment of diabetes [32]. Plants are rich sources of antidiabetic, anti-hyperlipidemic and antioxidant agents such as flavonoids, gallotannins, amino acids, and other related compounds.

Alpha Amylase Inhibition Assay: The digestion of starch takes place over several stages. Salivary amylase partially digests starch into shorter oligomers. This is followed by pancreatic amylase in the gut into maltose, maltotriose and small malto-oligosaccharides. Inhibition of alpha amylase can lead to reduction in post prandial hyperglycemia in diabetic condition. The IC_{50} values for the standard drug Acarbose and *T. arjuna* was found to be 324.81 (y = 0.0832x + 23.02, R^2 = 0.9072) and 210.91 (y = 0.072x + 34.35, R^2 = 0.9908) µg/ml respectively **Table 5, Fig 3A**. Our results showed that the bark extract of *T. arjuna* possess stronger antidiabetic activity when compared with acarbose standard. This shows that the bark of the medicinal plant *T. arjuna* could be a good alternative to other medicines used for treating diabetes.

Pancreatic Lipase Inhibition Assay: This enzyme is required for dietary triacylglycerol absorption catalyzing its hydrolysis to 2-monoacylglycerol and fatty acids prior to absorption. Natural compounds such as proteins and saponins are known to inhibit gastrointestinal lipases [30]. In addition, polyphenolic extracts from a number of plants have been shown to be effective inhibitors of the intestinal pancreatic lipase enzyme [36].

A dose-dependent manner of inhibition was exhibited by the bark extract of *T. arjuna* **Table 5**. Orlistat, the standard drug and a pancreatic lipase inhibitor, exhibited maximum inhibition of 75.71% at 500 g/ml while *T. arjuna* exhibited 66.45% at the same concentration. The IC_{50} values for Orlistat and *T. arjuna* was found to be 88.07 (y = 0.0587x + 44.83, R^2 = 0.9556) and 55.52 (y = 0.0458x + 47.64, R^2 = 0.48) µg/ml respectively **Fig. 3B**.

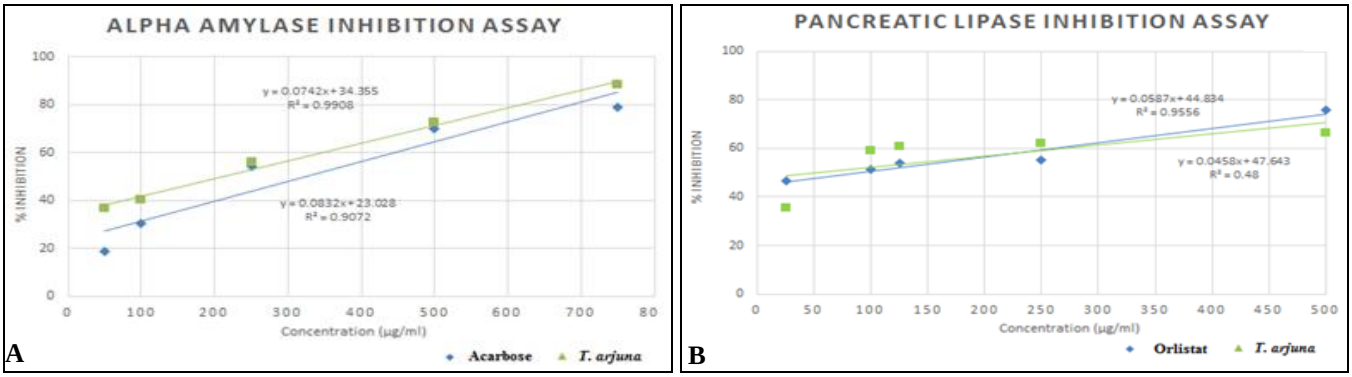


FIG. 3: ANTI-DIABETIC ACTIVITIES OF HYDROMETHANOLIC EXTRACT OF *T. ARJUNA* BARK; ALPHA AMYLASE INHIBITION ASSAY WITH ACARBOSE AS CONTROL (A) AND PANCREATIC LIPASE INHIBITION ASSAY WITH ORLISTAT AS CONTROL (B)

TABLE 5: EVALUATION OF *IN-VITRO* ANTI-DIABETIC ACTIVITIES OF HYDROMETHANOLIC EXTRACT OF *T. ARJUNA* BARK

Assay	Control			*T. arjuna* bark	
	Conc. (µg/ml)	% inhibition	IC_{50} (µg/ml)	% inhibition	IC_{50} (µg/ml)
Alpha amylase inhibition	50	18.9 ± 2.3	324.18	36.84 ± 5.1	210.91
	100	30.3 ± 5.6		40.22 ± 5.0	
	250	54.2 ± 9.3		56.06 ± 7.8	
	500	69.8 ± 11.2		72.59 ± 2.3	
	750	79.2 ± 9.9		88.46 ± 8.4	
Pancreatic lipase inhibition	25	46.73 ± 3.4	88.07	35.31 ± 0.7	51.52
	100	51.28 ± 2.8		59.20 ± 2.8	
	125	53.75 ± 9.9		61.00 ± 4.3	
	250	55.40 ± 7.4		62.11 ± 5.6	
	500	75.71 ± 5.6		66.45 ± 0.7	

Results are mean ± SEM (P < 0.05), obtained from three replicates.

***In-vitro* Glucose Adsorption Capacity:** The glucose adsorption capacity of the methanolic bark extract of *T. arjuna* at different glucose concentration was investigated in this study, and the results are presented in **Fig. 4**.

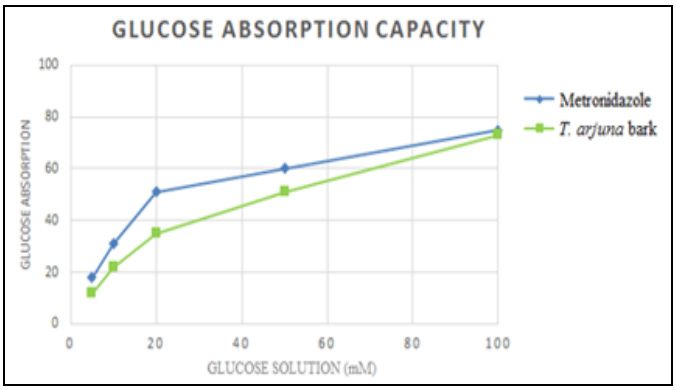


FIG. 4: GLUCOSE BINDING CAPACITY OF HYDRO-METHANOLIC EXTRACT OF BARK OF *T. ARJUNA* COMPARED TO THE CONTROL METRONIDAZOLE

It was observed that glucose binding capacity was directly proportional to the molar concentration of glucose. The bark sample was found to be effective in adsorbing glucose at both lower and higher concentrations. This could be attributed to the insoluble and soluble constituents and fibers present. Absorption of glucose would thus reduce the amount of glucose available for transport across the intestinal lumen, consequently reducing postprandial hyperglycemia event.

In-vitro* Glucose Diffusion Inhibitory Assay*: The methanolic extract of *T. arjuna* bark was subjected to this assay to determine glucose diffusion and GDRI across the dialysis membrane and the results are shown in **Table 6**. The rate of glucose diffusion across the membrane in the control was found to increase from 30 to 180 min however, inhibitory

effects on movement of glucose across the dialysis membrane was noted in the sample. GDRI was calculated to predict the effect of a fiber on the delay in glucose absorption in the gastrointestinal tract [37]. The GDRI was found to be a decrease in an increase with time. The higher GDRI value indicates higher retardation index of glucose by the sample. The inhibition in movement could occur due to the presence of fiber resulting in decrease in concentration and encapsulation of enzyme and starch.

TABLE 6: EFFECT OF HYDROMETHANOLIC EXTRACT OF *T. ARJUNA* BARK ON GLUCOSE DIFFUSION COMPARED TO THE CONTROL METRONIDAZOLE

Sample	Glucose content in dialysate (mM)			
	30 min	60 min	120 min	180 min
Control	3.50 ± 0.21 (53.1)	3.98 ± 0.18 (45.2)	5.02 ± 0.31 (22.5)	5.71 ± 0.51 (18.6)
T. arjuna	4.72 ± 0.11 (28.4)	4.09 ± 0.23 (16.6)	5.37 ± 0.33 (11.9)	4.70 ± 0.21 (9.2)

Values in parenthesis indicate GDRI. Values are expressed by mean ± SD of five samples in each group

Antimicrobial Activity of *T. arjuna*: Most of the bioactive medicinal metabolites are synthesized *via* plant secondary metabolic pathways during the vegetative stage of plant's life cycle and these compounds are responsible for the therapeutic properties of the plant tissue chosen. Chemical or synthetic drugs and antibiotics have been used for treatment of human diseases and infections. In general, these compounds effectively inhibit and/or stop microbial growth via disruption of the synthesis of microbial nucleic acids, proteins and cell walls [38]. It was found that all extracts of *T. arjuna* tested showed antibacterial activity against *S. aureus* with highest zone of inhibition in hot aqueous extract (28 mm) followed by cold aqueous extract (26 mm) **Table 7**. The zone of inhibition produced by the methanolic bark extract against *E. coli* was 17 mm, however, both hot and cold aqueous extracts lacked antibacterial activity. In general, except for *S. aureus*, it was observed that methanolic extract possessed slightly higher antibacterial activity than aqueous extracts. The limited spectrum of antimicrobial activity could be due to the polarity of antibacterial compounds which make them more readily extracted by methanol when compared to aqueous extract. In addition, antagonistic effects of other constituents may exert an influence on the bioactive compounds.

TABLE 7: ANTIBACTERIAL ACTIVITY OF HYDROMETHANOLIC EXTRACT OF *T. ARJUNA* BARK

Sample	Extract	Zone of inhibition (mm)			
		E. coli	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***
T. arjuna	Methanol	17 ± 0.5	13 ± 0.4	15 ± 0.5	24 ± 0.6
	Hot aqueous	-	7 ± 0.2	9 ± 0.4	28 ± 0.8
	Cold aqueous	-	6 ± 0.0	7 ± 0.1	26 ± 0.7
Control	Ciprofloxacin	37 ± 2.2	15 ± 0.6	31 ± 0.7	40 ± 1.2

All values are represented as mean ± SD (n = 3)

CONCLUSION: The present study, showed the presence of various bio active metabolites confirming it as a potent source for modern drugs. In addition, pharmacognostical characters and physicochemical parameters studied prove that the hydromethanolic extract of *T. arjuna* bark possesses potential antioxidant, anti-inflammatory, anti-diabetic and anti-microbial activities. Ethanomedicinal use of *T. arjuna* as a useful remedy in arthritic disorders could possibly be because of its excellent anti-inflammatory and antioxidant potential. These assays provide a scientific authentication to the traditional claims of its use in arthritis and other anti-inflammatory disorders. The hypoglycemic activity mediated by increasing glucose adsorption and decreasing glucose diffusion rate is suggestive of its anti-diabetic role. However, these results should be confirmed by *in-vivo* models and clinical trials for their effective utilization as therapeutic agents.

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