



Antioxidant and Hypoglycemic Activities of Leaf Extracts of Three Popular *Terminalia* Species

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Abstract: This study aimed to ascertain the antioxidant and hypoglycemic activity of methanolic extracts of the leaves of *Terminalia arjuna*, *T. bellerica*, and *T. chebula*. Extracts were evaluated for total phenolic, flavonoid, and tannin content, and *in vitro* antioxidant potential with DPPH, ORAC, and FRAP assays. The extracts' hypoglycemic activities were evaluated by hypoglycemic screening and an oral glucose tolerance test (OGTT) in normal rats. The methanolic extracts of *T. chebula* leaves exhibited the highest quantity of total phenolic and flavonoid content, followed by those of *T. bellerica* and *T. arjuna*. *T. arjuna* contained more tannin than *T. bellerica* did, but less than that of *T. chebula*. The scavenging capacity of *T. chebula* for the antioxidant DPPH was the highest of the extracts tested, as it recorded the lowest IC₅₀ value of all 3 extracts. Likewise, the results attributed the *T. chebula* extract with the highest oxygen radical absorption capacity (ORAC). In the FRAP assay, the extracts' ferric reducing antioxidant abilities were *T. arjuna* > *T. chebula* > *T. bellerica*. This correlates the potential of polyphenolic content enriched with antioxidant capabilities and substantiates the results of the hypoglycemic screening and OGTT, which determined that the *T. chebula* extract had a better hypoglycemic effect in normal and glucose-induced hyperglycemic rats ($p < 0.001$) than that of *T. bellerica* and *T. arjuna*, respectively. The use of these *Terminalia* species as food supplements may help in reducing oxidative stress and related diabetic complications. The phytoconstituents responsible for the hypoglycemic activity need to be isolated to elucidate the relationship between the extracts' antioxidant capacity and their hypoglycemic effects.

Keywords: Polyphenols, Antioxidant activity, Hypoglycemic activity, Oral glucose tolerance test, *Terminalia chebula*.

Introduction

The use of natural products as antioxidants in the management of diabetes mellitus has gained importance throughout the world. There has been increasing research focused on natural foods and medicinal plants and their phytoconstituents due to their well-known abilities to scavenge free radicals, *i.e.*, antioxidative ability¹⁻³. The interest in natural antioxidants has burgeoned since evidence of their potential interference in the production of reactive oxygen species (ROS) was uncovered, these ROS plays an important role in the progression of a great number of pathological disturbances such as inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, Alzheimer's disease, *etc*⁴⁻⁵.

The world health organization has also recommended and encouraged the use of natural products for the management of diabetes and in oxidative stress conditions, especially in countries where access to conventional treatment of diabetes is inadequate. There is an increased demand for natural products with antidiabetic activity due to the side effects associated with the use of insulin and oral hypoglycemic agents⁶.

Natural antioxidants such as flavonoids and polyphenols are believed to possess antioxidant properties due to their reducing and chelating capabilities. Flavonoids and polyphenols are secondary plant metabolites that are widely distributed in fruits, leaves, bark, and other parts in plants with free radical scavenging abilities⁷. The genus *Terminalia* (fam. Combretaceae), comprising 250 species, is distributed across tropical countries worldwide, and the Indian traditional system of medicine has documented several books and literatures on the medicinal values of many of its species, of which *Terminalia bellerica*, *T. chebula* and *T. arjuna* are prime examples^{8,9}. It has been reported that these species are rich in flavonoids and polyphenols. In "Ayurveda," a herbal formulation combining the dried fruits of *T. chebula*, *T. bellerica* and *Emblica officinalis* by the name of "Triphala" has been used as a food and dietary supplement to derive several health benefits such as laxation, detoxification, liver protection, anti-aging, and as a rejuvenator of the body¹⁰⁻¹¹. The combination has also been found to have antidiabetic and cholesterol-lowering activities¹²⁻¹³. The fruit extracts of *T. chebula* and *T. bellerica* have been shown to contain antioxidants¹⁴, and *T. arjuna* bark extracts have been reported to possess cardioprotective, antioxidant, and antimutagenic abilities¹⁵⁻¹⁷. Despite reports of the antioxidant and antidiabetic activities of the fruit and bark of *Terminalia* species, which are a rich source of flavonoids, tannins, and many phenolic derivatives, concrete evidence supporting the relationship with regard to the leaves of the genus is lacking.

Therefore, the present study was planned to investigate methanolic leaf extracts of the 3 popular species to determine their total phenolic (TPC), total flavonoid (TFC), and total tannin content (TTC), as well as their 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, oxygen radical antioxidant capacity (ORAC), and ferric reducing antioxidant power (FRAP) in correlation to their hypoglycemic activity.

Experimental

Dried powdered leaves of *T. arjuna*, *T. bellerica*, and *T. chebula* were obtained from Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd, Jogli, India. These samples were successively defatted with hexane, then chloroform, and finally with methanol using a

Soxhlet apparatus. The resulting methanol extracts were evaporated under reduced pressure at 40°C using a rotary evaporator to derive a crude methanol (MeOH) extract that was further lyophilized and stored at -20°C prior to use.

Determination of Total Phenolic and Flavonoid Content

The TPC and TFC of the methanolic extracts were determined by the Folin-Ciocalteu and Dowd methods as adapted by Lamien-Meda *et al*¹⁸. Results are expressed as mg Gallic Acid equivalents (GAE) and mg Quercetin (Q) equivalents.

Determination of Total Tannin Content

The TTC in the methanolic extracts was estimated with the method by Price and Butler¹⁹ with some modifications. A 3.0 mL volume of the sample, 3.0 mL of vanillin (4%) in methanol and 1.5 mL con. HCl were mixed and were incubated in the dark for 10 min. Subsequently, the TTC content of the samples were analyzed with a UV-Vis. spectrophotometer at 500 nm. Results are expressed as mg Catechin (C) equivalents.

In vitro antioxidant study

DPPH Radical Scavenging Activity

The scavenging activity of all the 3 methanolic extracts on DPPH (1,1-diphenyl-2-picrylhydrazyl) was determined using the method described by Choi *et al*²⁰ with slight modifications. This method is based on the reduction of purple DPPH to a yellow colored diphenylpicrylhydrazine. Changes in color were measured at 518 nm. All the extracts were tested at final concentrations ranging 600–10 µg/mL in ethanol. One milliliter of 0.3 mM DPPH ethanol solution was added to 2.5 mL of sample solution in different concentrations to produce the test solutions, while 1 mL of ethanol was added to 2.5 mL of sample to produce the blank solutions. The negative control consisted of 1 mL of DPPH solution plus 2.5 mL of ethanol. The solutions were allowed to react at room temperature for 30 min in the dark. The absorbance values were measured at 518 nm and converted into percentage antioxidant activity using the following equation:

$$\% \text{ Inhibition} = [(AB - AA)/AB] \times 100,$$

where AB: absorption of blank sample; AA: absorption of tested samples. The half maximal inhibitory concentration (IC₅₀) and the kinetics of DPPH scavenging activity were determined. Ascorbic acid and butylated hydroxytoluene (BHT) were used as positive controls in this assay.

ORAC Antioxidant Activity Assay

The oxygen radical absorbance capacity (ORAC) assay was carried out based on the procedure described by Cao G *et al*²¹ with slight modifications. Briefly, 175 µL of the sample/blank were dissolved with PBS at concentrations of 160 µg/mL, pH 7.4. Serial dilutions of the standard Trolox were prepared from 75 mM. The assay was performed in 96-well black microplates, to which 25 µL each of samples (extracts), standard (Trolox), blank (solvent/PBS), or positive control (quercetin) were added. Subsequently, 150 µL of fluorescent sodium salt solution was added, and the plate was incubated for 45 minutes at 37°C. 2,20-azobis (2-amidinopropane) dihydrochloride (AAPH) solution (25 µL) was added to make up a total volume of 200 µL/well. Fluorescence was recorded at 37°C until it

reached 0 (excitation at 485 nm, emission at 535 nm) using a fluorescence spectrophotometer (Perkin-Elmer LS 55) equipped with an automatic thermostatic autocell-holder. Data were collected every 2 min for 2 h and were analyzed by calculating the differences of areas under the fluorescein decay curve (AUC) between the blank and the sample. Values are expressed as Trolox equivalents.

FRAP Assay

The FRAP (ferric reducing/antioxidant power) assay was modified from the method used by Benzie and Strain²². The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM FeCl₃·6H₂O solution. A fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL FeCl₃·6H₂O. The temperature of the solution was raised to 37°C before use. Extracts (10 µL) were allowed to react with 190 µL of FRAP solution for 30 min in the dark. Colorimetric readings of the product, *i.e.*, the ferrous-TPTZ complex, were taken at 593 nm for 10 min and a steady state was reached within 5 min for the different test substance concentrations. The EC₁ value was calculated from the regression curve as the concentration of antioxidant (IM) giving an absorbance reading equivalent to that obtained with a 1 mM Fe(II) solution. The standard curve was linear between 200 and 1000 µM FeSO₄. Results are expressed as µM Fe (II)/g dry mass and compared with those of ascorbic acid and BHT.

In Vivo Study

Animals and Treatment

Male Sprague-Dawley rats weighing 200–250 g were procured from the Animal Care Unit of the University Malaya Medical Centre (UMMC) in Kuala Lumpur, Malaysia and maintained under pathogen-free conditions in the animal housing unit in a temperature-(23±2°C) and light-controlled (12 h light/dark cycle) room with 35–60% humidity. Animals were provided with carbohydrate-enriched rodent chow and water *ad libitum*. The Animal Ethics Committee from the UMMC Faculty of Medicine approved the study under Approval No. FAR/10/11/2008/AA(R).

Acute Oral Toxicity

The acute oral toxicity test of the 3 plants' methanolic extracts was planned according to the Organization for Economic Co-operation and Development (OECD) guidelines. Healthy male Sprague-Dawley rats weighing 150–200 g were used for this study. After an overnight fast, the rats were divided into 6 groups (n = 9–10) and orally fed with the methanolic leaf extracts. Three groups were fed with doses of 50 mg/kg body weight and the other 3 groups were fed doses of 2500 mg/kg body weight. The rats were observed continuously for 24 h for any behavioral, neurological, and autonomic profiles, and after 72 h for any lethality. According to this toxicity study, 1/10 of the maximum dose administered was used for the hypoglycemic screening study.

Determination of Postprandial Blood Glucose Levels in Normal Rats

Animals were divided into 5 groups of 8–9 in a group. One group was used as a positive control and received glibenclamide (5 mg/kg) orally; the negative control group received NaCl (0.9%, dose 2 mL/200 g), while the other 3 groups received methanolic leaf extracts (250 mg/kg). The lyophilized powder of all leaf extracts was reconstituted with filtered water and administered orally using a gavage (2 mL/200 g body weight). Blood samples were obtained by amputation of the tail tip in non-fasting conditions after 2, 4 and 6 h of treatment. Glycemia was determined using the glucose oxidase-peroxidase enzymatic method (Accu-Check Performa, Roche Diagnostic Germany) through electronic glucometer.

An extract was deemed to have a hypoglycemic effect if the blood glucose levels of the rats decreased significantly compared to those of the normal control group²³.

Glucose Challenge (Glucose Tolerance Test)

An oral glucose tolerance test (OGTT) was used to evaluate the effectiveness of the leaf extracts with hypoglycemic potential on glucose induced hyperglycemic rats, which was derived from prior screening tests and from which the positive results formed the basis of this hypoglycemic screening. In rats fasted overnight, extracts (250 mg/kg) were fed in doses of 2.0 mL/200 g body weight and the control group was given NaCl (0.9%). Concentration of glucose level in blood was measured before and 120 min after extracts administration. After this time point, glucose (3 g/kg) was introduced orally, then glucose concentration in blood was measured at 30, 60 and 120 min post-challenge²³.

Statistical Analysis

The data are expressed as the mean \pm SD of 3 measurements. Statistical analysis was performed using one-way and two-way repeated measures ANOVA and comparison of the data was done by Tukey's test with all pair-wise multiple comparison procedures. $p < 0.001$ was considered statistically significant.

Results and Discussion

In this study, we evaluated the total phenolic, total flavonoid, and total tannin content in the methanolic extract of *T. chebula*, *T. bellerica* and *T. arjuna* leaves, followed by evaluation of the extracts' antioxidant activities and *in vivo* hypoglycemic screening in normal rats.

Total Phenolic, Flavonoid and Tannin Content

There has been an increased desire in consumers for functional foods with antioxidant capabilities. The fruit and leafy parts of plants are considered rich in polyphenols and flavonoids, which contributes to their antioxidant capacity²⁴. The antioxidant property of phenolic compounds is attributed to their ability to absorb and neutralize free radicals.

The results in Table 1 demonstrate that the methanolic extract of *T. chebula* leaves contained the highest TPC, followed by those of *T. bellerica* and *T. arjuna* (266.16, 259.28, and 147.23 mg GAE/g extract, respectively), likewise, the TFC were 29.23, 16.15, and 8.19 mg Q/g extract, respectively. *T. chebula* possessed the highest TTC, followed by *T. arjuna* and *T. bellerica* (8.36, 4.68 and 6.31 mg C/g extract, respectively). *T. chebula* fruits have been found to contain higher amounts of total phenolics, total flavonoids and total tannin, and are reported to contain gallic acid, chebulic acid, 1,6-di-*O*-galloyl- β -*D*-glucose, punicalagin, 3,4,6-tri-*O*-galloyl- β -*D*-glucose, casuarinin, chebulanin, chebulagic acid, chebulinic acid and 1,2,3,4,6-penta-*O*-galloyl- β -*D*-glucosein, which might be responsible for its high antioxidant activity²⁵.

Table 1. Total phenolic, flavonoid, and tannin content in methanolic extracts of *Terminalia chebula*, *T. bellerica*, and *T. arjuna* leaves.

No.	Plants	Total Phenolic Content ^a (mg GAE/g extract)	Total Flavonoid Content ^a (mg Quercetin/g extract)	Total Tannin Content ^a (mg Catechin/g extract)
1	<i>T. chebula</i>	266.16 \pm 7.81	29.23 \pm 3.81	8.36 \pm 0.37
2	<i>T. bellerica</i>	259.28 \pm 6.42	16.15 \pm 2.42	4.68 \pm 1.31
3	<i>T. arjuna</i>	147.23 \pm 2.34	8.19 \pm 1.41	6.31 \pm 0.17

^aValues expressed are mean \pm SD of triplicate measurements.

In Vitro Antioxidant Activities

The antioxidant activities of the *T. chebula*, *T. bellerica* and *T. arjuna* leaf extracts were determined with DPPH, ORAC and FRAP assays. These assays were measured in triplicate at different concentrations to determine the extracts' IC₅₀ values. The results are listed in Table 2.

DPPH Radical Scavenging Activity

The DPPH radical is used commonly and extensively to determine the *in vitro* antioxidative activity of antioxidant compounds. When treated with substances or samples that are hydrogen atom donors, the DPPH radical is converted into a stable DPPH radical, indicated by a color change from purple to yellow (Kriengsak Thaipong, 2006). DPPH radical reduction is analyzed by measuring the absorbance of samples at a 518 nm wavelength. The DPPH scavenging capacity of *T. chebula* (11.6 µg/mL) was the highest of the 3 extracts tested, as it recorded the lowest IC₅₀ value of all the extracts, while *T. bellerica* (16.4 µg/mL) demonstrated better scavenging activity than *T. arjuna* (21.8 µg/mL) did. Among the positive controls, ascorbic acid (1.6 µg/mL) was found to have better DPPH radical scavenging ability than BHT (1.7 µg/mL). Flavonoids are well-known antioxidant constituents of plants and possess a broad spectrum of chemical and biological activities, including radical-scavenging or chelating activities²⁶.

ORAC Assay

All the extracts demonstrated good oxygen radical absorption capacity; IC₅₀ values are expressed in Trolox (µM/mL) equivalent (TE) concentrations as listed in Table 2. *T. chebula* exhibited lower IC₅₀ values (18.23 µM TE/mL) than the other 2 species did. *T. arjuna* had an IC₅₀ value of 42.31 µM TE/mL, whereas that of *T. bellerica* was 29.17 µM TE/mL. The positive control (Quercetin) had the lowest IC₅₀ value (12.16 µM TE/mL). Therefore, the results attribute *T. chebula* extracts with the highest oxygen radical absorption capacity. The ORAC assay is based upon the inhibition of the peroxy-radical-induced oxidation initiated by thermal decomposition of azo-compounds such as [2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)].

Reactive oxygen species (ROS) are hazardous to cellular structures and functional molecules (*i.e.* DNA, proteins, lipids) as they act as strong oxidizing mediators or free radicals. Biological antioxidants are able to dispose of ROS; as they are found to be safe and effective in eliminating all of the free radicals, oxygen ions and peroxides that can do damage to the body⁷. In this manner, the ORAC assay uses a biological relevant radical source and it combines both inhibition time and degree of inhibition into one quantity²¹.

FRAP Assay

The principle of a FRAP assay involves the reduction of ferric ions to ferrous ions by plant extracts due to the presence of reducing substances in the extracts²⁷. The extracts' ability to reduce ferric ions was determined using the FRAP assay developed by Benzie and Strain¹⁰. Electron-donating antioxidant compounds are capable of reducing the ferric-TPTZ (Fe(III)-TPTZ) complex to a blue ferrous-TPTZ (Fe(II)-TPTZ) complex that exhibits strong absorbance at 593 nm. Evaluation of the extracts' ferric reducing antioxidant ability determined that the EC₁ value of *T. arjuna* (232 µg/mL) was lower than that of *T. chebula* (243 µg/mL) and *T. bellerica* (265 µg/mL). The lower the EC₁ value, the higher the ferric reducing antioxidant abilities of the extract. The BHT (3.2 µg/mL) standard had greater ferric ion reducing capability than ascorbic acid (3.7 µg/mL) did. The reducing capacity of *T. arjuna* leaf extract may have been due to a large number of polyphenolic compounds with electron-donating hydroxyl groups.

Table 2. Antioxidant activities of *Terminalia chebula*, *T. bellerica* and *T. arjuna* methanolic leaf extracts in DPPH, ORAC and FRAP assays.

No.	Samples	^a DPPH IC ₅₀ , μg/mL	^a ORAC IC ₅₀ , μM/mL	^a FRAP EC ₁ , μg/mL
1	<i>T. chebula</i>	11.6±0.43	18.23±0.9	243±5.8
2	<i>T. bellerica</i>	16.4±0.55	29.17±1.8	265±6.7
3	<i>T. arjuna</i>	21.8±0.37	42.31±2.7	232±8.3
4	Ascorbic acid	1.6±0.14	Not applicable	3.7±0.8
5	BHT	1.7±0.07	Not applicable	3.2±0.5
6	Quercetin	Not applicable	12.16±1.3	Not applicable

ORAC: Equivalent conc. Trolox (20 μg/mL) (μM) ^aDPPH, ^aORAC and ^aFRAP assays demonstrating low values indicate high antioxidant activity. Standard deviation (SD) values of a minimum of 3 replicates.

***In Vivo* Study**

Acute Oral Toxicity Studies

The leaf extracts of the 3 selected species were found to be non-toxic in rats, as neither mortality nor any considerable symptoms of toxicity were observed after oral administration of extracts at 2 dose levels (50 and 2500 mg/kg body weight) over a 72-hr period.

Hypoglycemic Activity in Methanolic Leaf Extracts by Oral Route

There was a reduction in blood glucose levels at 2 and 4 hr, with a slight increase at 6 h after oral administration of *T. chebula*, *T. bellerica* and *T. arjuna* leaf extracts. After glibenclamide administration, blood glucose levels were significantly reduced at 2, 4, as well as 6 h, but blood glucose levels did not change significantly within the different time intervals after oral administration of the negative control (0.9% NaCl). The results are listed in Table 3. There was a significant difference in blood glucose levels at 2, 4 and 6 h after oral administration of glibenclamide, *T. chebula*, *T. bellerica*, and *T. arjuna* extracts ($p < 0.001$) as compared to levels at 0 h and in the negative control (NaCl) group.

The control of postprandial hyperglycemia is one of the beneficial therapies for management of type 2 diabetes mellitus²⁸, along with nutrition and oral hypoglycemic and insulin therapies²⁹. In order to determine a scientific basis for the utilization of *T. chebula*, *T. bellerica* and *T. arjuna* leaves in the treatment of diabetes, we decided to evaluate their extracts' hypoglycemic effect in normal rats. Earlier reports revealed that *T. chebula* and *T. bellerica* fruits and *T. arjuna* bark possessed antidiabetic effects when studied in STZ-induced diabetic animals, and suggested that most diabetic complications were mediated through oxidative stress³⁰. Production of ROS and its related oxidative stress was reported as the root cause for the development of insulin resistance, β -cell dysfunction, impaired glucose tolerance and type 2 diabetes mellitus³¹. The present study demonstrated that *T. chebula* leaf extract was more effective at decreasing blood glucose in normal rats, followed by *T. bellerica* and *T. arjuna* when compared to the negative control. This may be due to their flavonoid and phenolic contents, which are known to be involved in the healing process of free radical-mediated diseases, including diabetes³².

Table 3. Hypoglycemic activity of methanolic leaf extracts by oral route in normal rats.

Group	Alteration in blood glucose levels (mmol/L) before and after administration of test samples (mean \pm SD)				
	0 h	2 h	4 h	6 h	<i>p</i> -value
NaCl control	7.5 \pm 0.32	7.1 \pm 0.23	6.6 \pm 0.37	6.7 \pm 0.28	0.059
Glibenclamide	7.2 \pm 0.41	4.6 \pm 0.33*	4.5 \pm 0.24*	4.1 \pm 0.45*	<0.001
<i>T. chebula</i>	6.9 \pm 0.37	5.4 \pm 0.26*	4.9 \pm 0.52*	5.1 \pm 0.31*	<0.001
<i>T. bellerica</i>	6.6 \pm 0.29	4.9 \pm 0.17*	5.1 \pm 0.37*	5.4 \pm 0.46*	<0.001
<i>T. arjuna</i>	7.0 \pm 0.36	5.8 \pm 0.24*	5.2 \pm 0.38*	5.7 \pm 0.29*	<0.001

*Comparison with time 0 and negative control (NaCl) indicates significance ($p < 0.001$).

Oral Glucose Tolerance Test

An oral glucose tolerance test was used to confirm the result of the hypoglycemic effects. The results depicted in Fig 1. indicate that there was no major alteration in blood glucose levels until 120 min following administration of extracts and NaCl in overnight-fasted normal rats. However, after 30 min of glucose load, blood glucose levels were considerably elevated in all treated groups up until 60 min, followed by a fall in glucose concentration until 120 min. Hence, there was a significant difference in blood glucose levels throughout the observation period for all 3 leaf extracts ($p < 0.001$ when compared with the normal control group). This suggests that methanolic leaf extracts of *Terminalia* species have the ability to improve glucose tolerance activity in normal rats. This observation indicates that the extracts may interfere with the intestinal glucose absorption in the gut by various mechanism, this may be postulated that the positive results of these extracts might stimulates glycogenesis in the liver, which is enhanced by feeding and is hypothesized that the glucose tolerance activity induced by the extracts could be due to a mixture of active compounds that reduce the concentration of glucose and improve glucose tolerance activity³³⁻³⁴.

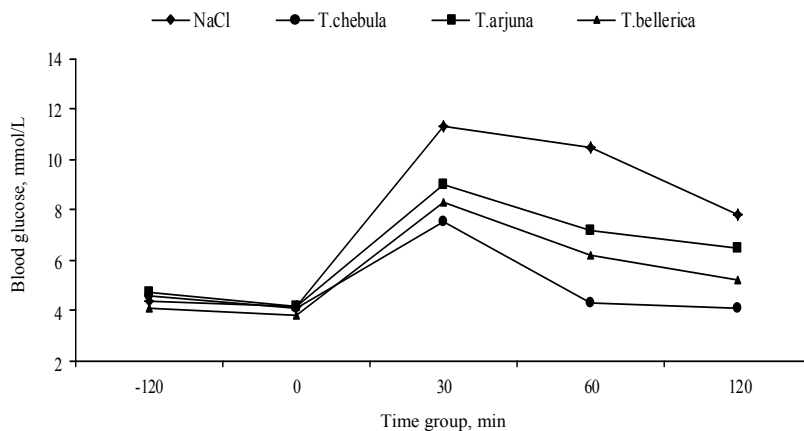


Figure 1. In normal fasted rats blood glucose levels, significantly changed at different time intervals after oral administration of extracts and glucose load ($p < 0.001$).

Conclusion

The present study indicates that the leaf extracts of the selected *Terminalia* species possess antioxidant and hypoglycemic activities, which is probably due to their phenolic groups, and

brings new hope to research on the management of type 2 diabetic conditions. The use of these species as food supplements may aid in reduction of oxidative stress and related diabetic complications. However, the mechanism of action by which these species exert their action needs to be established by a thorough phytochemical investigation to identify the constituents responsible for the antioxidant and hypoglycemic activity. Furthermore, long-term studies on type 2 diabetic models must be carried out to describe the exact mechanism of action at the cellular level.

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