Antimicrobial and Antidiabetic activities of different parts of *Terminalia bellerica* fruits.

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Abstract:

Terminalia bellerica (Gaertn.) Roxb. which is also known as baheda or bahera it is one of the most deciduous trees in the Combretaceae family. It is used for the preparation of triphala, an Indian herb. The present study ~~was~~ aimed to compare phytochemicals, antimicrobial activity and antidiabetic activity activities in pulp, seed and seedcoat of *T. bellerica* collected from Mohali, Punjab. Extracts of different solvents, viz., hexane, methanol and aqueous were prepared using cold maceration method. Preliminary phytochemical analysis revealed the presence of most of the phytochemicals in all the parts, however, alkaloids were absent in all solvent extracts of seeds. Phenols were found to be absent in hexane extract of pulp and seed. Total phenolic content was found to be highest in aqueous extract of seed coat (154.22±2.33 mg/g GAE) and methanol extract of seed (119.06±6.65 mg/g GAE). Among all extracts, methanolic extract of seed coat showed lowest MIC (3.125 µg/mL) against both tested gram-positive bacteria (*B. subtilis*, and *S. aureus*), while, methanolic pulp extract was found to be more effective against *K. pneumoniae* and *P. aeruginosa* with MIC of 3.125 µg/mL. Hexane extract of pulp showed higher inhibitory activity against *E. coli* with least MIC (3.125 µg/mL). Aqueous extract of seed coat showed strong in vitro antidiabetic activity with IC_{50} value of 3.92 µg/mL as compared to that of standard drug, metformin (14.5 µg/mL). The results of the present study showed therapeutic applications of seed coat of *T. bellerica fruits.* However, further studies are needed to isolate the phytocompounds responsible for their biological activities.

Keywords: *Terminalia bellerica;* Fruits; Extractions; Total Phenols; Antimicrobial; Antidiabetic.

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Introduction:

For millennia, medicinal plants have been an integral part of both traditional and modern medicine (Thirumalai and al. 2009; Saini and al. 2022). A significant portion of the population relies on medicines made from natural ingredients since they are risk-free, efficient, and simple to get (Azaizeh and al. 2003; Katewa and al. 2004). As a result, medicinal plants have become an integral component of the human health care system (WHO 2002). Furthermore, medicinal plants have received increased attention due to their effectiveness, the increased expense of conventional drugs, and cultural preferences (Tabuti and al. 2003). Because of the advent of multiple drug resistance and the adverse effects of synthetic medications, there has been a remarkable surge in the hunt for powerful antimicrobials and antioxidants that are derived from plants (Sharma and al. 2012). Additionally, phenolic compounds found in plants act as powerful antioxidants to scavenge free radicals and donate hydrogen to cells, thereby protecting them from several diseases (Chew and al. 2011). Diabetes is one of the serious metabolic disorders that affect millions of live (Joshi, and Parikh 2007; Kumar and al. 2013). Previous studies revealed that diabetes-related oxidative stress led to various macro and microvascular problems (Kawahito and al. 2009; Ferdinando and Michael 2010). Most of the traditional medicines used to treat diabetes are ineffective and have serious side effects (Gupta and al. 2016). These constraints have motivated the use of medicinal plants as cost-effective antidiabetic medicines with fewer adverse effects (Atanasov and al. 2015).

Terminalia is a genus of large trees (upto 40 m high) of the flowering plant family Combretaceae, comprising around 200 species distributed across humid, semi-humid, tropical regions of the world. Approximately 24 different species of *Terminalia* have already been reported from various states and Union Territories of India (Srivastav 2003). *Terminalia bellerica* Roxb. (Combretaceae) is a large deciduous tree that is abundant in the moist valleys of India and its fruits are frequently used in Indian traditional medicinal system (Chopra and al. 1956). Fruits of *T. bellerica* are one of the ingredients of “Triphala”, an Ayurvedic formulation that is believed to encourage health, immunity and long life (Tarasiuk and al. 2018). Several bioactive compounds such as β-sitosterol, gallic acid, ellagic acid, ethyl gallate, chebulagic acid, arjungenin, belleric acid, bellericoside have been reported from fruits of *T. bellerica* (Dhanani and al. 2014; Fahmy and al. 2015; Sobeh and al. 2019; Gupta

and al. 2020). Fruits are useful in the treatment of asthma, bronchitis, hepatitis, diarrhea, piles, dyspepsia, eye diseases, hoarseness of voice, and scorpion-sting and also used as hair tonic (Rastogi and Mehrotra 2004; Singh 2011). Decoction of the green fruit is useful in the treatment of cough (Deb and al. 2016). Fruit pulp is used in dysenteric-diarrhea, leprosy, piles, and dropsy (Singh and al. 2018). Partially ripe fruit acts as purgative and fruit kernel are narcotic. *T. bellirica* fruit is used in the treatment of menstrual disorder in Bangladesh. Appreciable antimicrobial activity has been associated with the triterpenoid compounds present in the fruits (Deb and al. 2016). Bark gum and kernel oil show purgative actions, while seed oil exhibits anti-rheumatic activity (Ghani 2003). The leaves enhance appetite, relieve piles, lower cholesterol and blood pressure, boost immunity and prevent ageing and also improve the body's ability to resist against pathogens (Kumar and Khurana 2018). Keeping in mind the vast importance of *T. bellerica* fruits, the present study was aimed to compare the phytochemical composition, and biological activities such as antimicrobial and antidiabetic activity in various solvent extracts of different parts of *T. bellerica* fruits.

Materials and Methods:

Chemicals and Solvents

All of the chemicals used in this study were of analytical grade. Nutrient broth used for antibacterial activity was obtained from Himedia Labs, Mumbai. The chemicals such as DNS (dinitrosalicylic acid), Folin-ciocaltaeu reagent, sodium potassium tartrate, mono sodium phosphate, disodium phosphate, and solvents such as n-hexane, methanol, dimethyl sulphoxide (DMSO) etc. were obtained from Loba Chemie Pvt. Ltd., Mumbai. Gallic acid was purchased from Himedia Labs, Mumbai.

Microbial Strains

Bacterial strains (*Bacillus subtilis*, MTCC441, *Staphylococcus aureus*, ATCC25922; *Escherichia coli*, ATCC29213; *Klebsiella pneumoniae*, MTCC39 and *Pseudomonas aeruginosa*, MTCC424), used in this study were obtained from Institute of microbial technology (IMTECH), Chandigarh. Bacterial strains were maintained at 35±2° C on nutrient agar (NA) media.

Collection and Preparation of samples

The authentic fruit samples of *Terminalia bellerica* were collected from herbarium garden of Mohali region of Punjab, India. The collected samples were cleaned properly and washed by running tap water to remove the dust and remaining residue and then different parts of fruits such as pulp, seed coat, and seed were separated. All the parts were completely dried in an incubator at 37 °C for 24-48h and then converted to fine powder using an electric grinder. The fine powders of different parts were stored in tight glass containers for the further research.

Preparation of extracts

The dried powder of each part of fruit (5 g) of *T. bellerica* were cold macerated in n-hexane, methanol and autoclaved distilled water (50 mL) in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 120 rpm for a week to ensure complete extraction. The extracts were filtered through Whatman No.1 filter paper and then centrifuged (4000× g for 5 min). The supernatant was collected and allowed to evaporate at 40 ^{o}C to yield thick and viscous residues as crude extracts. The dried crude extracts were stored at 4 °C in air-tight bottles till use (Kumar and al. 2018).

Qualitative analysis of phytochemicals

Phytochemicals play an important role in the treatment of different types of diseases and disorders and are still used in both traditional and modern medicine. Different phytochemical tests for the detection of various phytocompounds such as phenolics, tannins, flavonoids, phytosteroids and saponins were used as per standard methods (Khandelwal 2007; Guleria and al. 2016; Kumar and al. 2021).

Quantitative analysis of total phenols (TPC)

For the analysis of TPC of sample extracts, Folin ciocalteau reagent method was used (Singleton and al. 1999; Chandel and al. 2019; Guleria and al. 2020; Sharma and al. 2021; Gautam and al. 2021). The amount of TPC was calculated from calibration curve of gallic acid (5-100 µg/ml) and expressed in terms of gallic acid equivalents (GAE) per gram of extract using following equation-

$$TPC = (c \times V)/m$$

Where TPC is total content of phenolic compounds, mg/g plant extract, in GAE, c is the concentration of gallic acid established from the calibration curve (mg/ml), V is the volume of extract in ml and m is the weight of crude plant extract in g.

Antimicrobial activity in fruit extract of Terminalia bellerica by using broth dilution method

The minimum inhibitory concentration was analyzed by most widely accepted serial dilution microplate procedure using ampicillin as positive control (Kumar and al. 2018; Sharma and al. 2022). In this method, extracts of different solvents (100 µl) (1mg/ml, w/v) were serially diluted using nutrient broth and then 10 µl bacterial culture (OD_{600}~0.2) was added to each well. DMSO was used as solvent control in both cases. The micro plates were incubated 37°C for overnight. After incubation, 2,3,5-triphenyterrazolium chloride dye (10 µl of 5 mg/ml) was added to each well and again incubated at 37°C for 2 h. The change in the color was observed visually. The MIC was determined by change in the color of dye from purple to pink or colorless. The lowest concentration at which color change was taken as the MIC value.

Determination of in vitro antidiabetic activity

Anti-diabetic potential of different parts of *T. bellerica* fruits was evaluated using *in vitro* α-amylase inhibition method. In this procedure, the enzyme solution was prepared by dissolving α-amylase in 20 mM phosphate buffer (pH-6.9) at the concentration of 0.5 mg/ml. The reaction mixture containing 1 ml of extracts of various concentrations (20, 40, 60, 80 µg/ml) was then incubated at 25°C for 10 minutes with the addition of 1mL of starch (0.5%) solution. The reaction was terminated by addition of 2 mL of dinitrosalicylic acid (DNS, color reagent), and heating the reaction mixture in a boiling water bath for 5 min. The absorbance was measured at 540 nm using spectrophotometer (Subramanian and al. 2008; Aljarah and Hameed 2018). Metformin (20-80 µg/mL) was used as standard drug in this experiment. The inhibition percentage was calculated using the given formula-

Percentage inhibition = $A_{Control} - A_{Sample} \times 100/A_{Control}$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} is the absorbance of the test sample. The experiment was performed in duplicated and results were calculated as mean ± S.D.

Results and Discussions:

Preliminary phytochemical analysis:

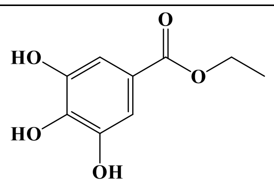
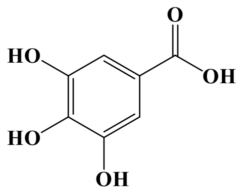
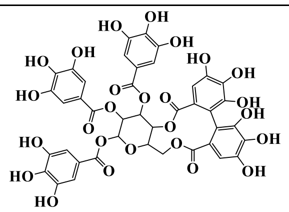
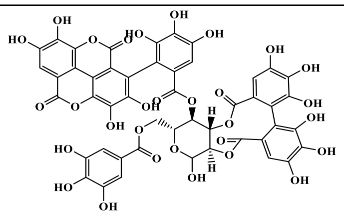
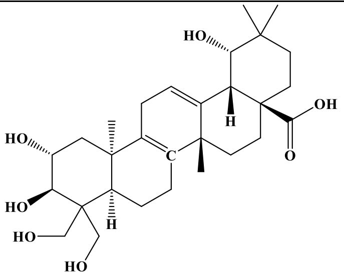
The results of qualitative analysis of solvent extracts of various parts of *T. bellerica* fruits are listed in Table-1. Most of the phytochemicals were found to be present in all the parts, however, alkaloids are absent in all solvent extracts of seeds. Phenols were found to be absent in hexane extract of pulp and seed. All the parts of *T. bellerica* fruits lack saponins and proteins. Similar to our study, Hazra (2019) have also compared the phytochemicals of various parts of *T. bellerica* fruits from Kolkata, West Bengal and reported the presence of most of the secondary metabolites in epicarp and mesocarp of fruit. The presence of alkaloids, phenol, tannins and flavonoids in different solvent extracts of *T. bellerica* fruits collected from Coimbatore, Tamil Nadu was reported by Devi and al. (2014). Study from Chandel and al. (2016) have reported the presence of alkaloids, phenolics, tannins, flavonoids, carbohydrates, glycosides, phytosterols, phytosteroids, saponins, and proteins in ethanolic extract of *T. bellerica* fruits from Himachal Pradesh, India. Some of the important phytocompounds reported in fruits of *T. bellerica* were listed in table-2.

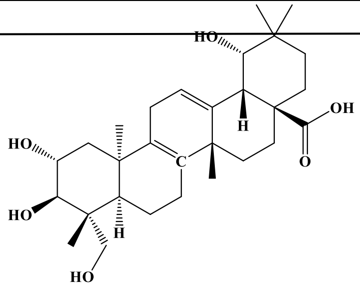
Table-1: Qualitative analysis of phytochemicals in various parts of *T. bellerica* fruits.

Phytocompounds	Pulp			Seed coat			Seed		
	Aq	Meth	Hex	Aq	Meth	Hex	Aq	Meth	Hex
Alkaloids	+	+	+	+	+	+	-	-	-
Phenols	+	+	-	+	+	+	+	+	-
Flavonoids	+	+	+	+	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+	+	+	+
Saponin	-	-	-	-	-	-	-	-	-
Proteins	-	-	-	-	-	-	-	-	-
Phytosteroids	+	+	+	+	+	+	+	+	+

Aq-Aqueous extract, Meth-Methanol extract, Hex-Hexane extract. (+) indicate presence, while (-) indicates absence of phytochemicals.

Table-2: Summary showing pharmacological properties of the major compounds from *T. bellerica* fruits.

Compound name	Structure	Pharmacological Properties	References
Ethyl gallate		Antimicrobial, antioxidant, anti-inflammatory activity	Pfundstein and al. 2010)
Gallic acid		Antibacterial, anti-inflammatory, antimutagenic, cardioprotective, antioxidant, anticancer, hepatoprotective, neuroprotective activity, antidiabetic activity	Jadon and al. 2007; Latha and Daisy 2011
Tellimagrandin II		Antioxidant, antimicrobial, anti –inflammatory activity	Pfundstein and al. 2010
Terflavin B		Anti-HIV-1, antimalarial, and antifungal activity	Pfundstein and al. 2010
Bellericagenin A		Antimicrobial activity, antioxidant activity	Mahato and al.1992

Arjungenin		Cardioprotective, Antioxidant	Varghese and al. 2015; Mohanty and al. 2019

Variation in the total phenolic contents in fruit extracts of T. bellerica

The extract of *T. bellerica* was further estimated by using spectrophotometric methods of total phenolics illustrated in Fig-1. Total phenolic content revealed to be highest in aqueous extract of seed coat (154.22±2.33 mg/g GAE) followed by methanol extract of seed (119.06±6.65 mg/g GAE). Least amounts of phenols were found to be present in hexane extract of pulp (21.63±1.81 mg/g GAE), seed (20.77±1.63 mg/g GAE), and seed coat (8.06±1.46 mg/g GAE) (Fig. 1). Study from Hazra (2019) have showed the presence of higher TPC in epicarp (139±0.09 mg GAE per gram dry weight) and mesocarp (135±0.12 mg GAE per gram dry weight) as compared to that of seed (0.65±0.02 mg GAE per gram dry weight) of *T. bellerica* fruits from Kolkata, West Bengal, India. Chandel and al. (2016) have also showed higher amount of TPC in ethanolic extract of whole fruits (177±0.19532 mg/g GAE) from Himachal Pradesh, India.

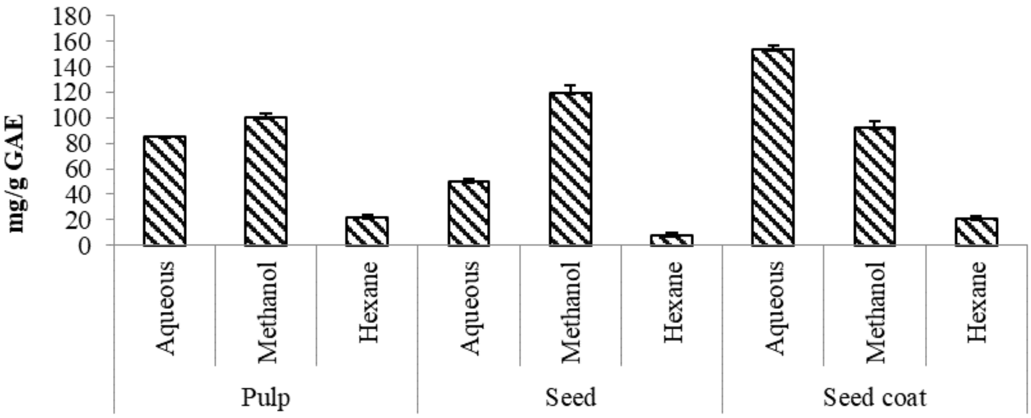


Fig:1: Variation of phenolic content in various parts of *Terminalia bellerica* fruit extracts.
Values were calculated from standard curve of gallic acid and were expressed as mg/g GAE. The values are mean±S.D. of two experiments.

Analysis of antimicrobial activity using broth dilution method

Broth dilution method was used to analyze the lowest concentration of the assay antimicrobial agent (minimum inhibitory concentration, MIC) that inhibits the visible growth of the bacterium which is being examined under defined test condition. The results of broth dilution were expressed in table-2 and it was observed that among all extracts, methanolic extract of seed coat showed lowest MIC (3.125 µg/mL) against both tested gram-positive bacteria (*B. subtilis*, and *S. aureus*). Methanolic pulp extract was found to be more effective against *K. pneumoniae* and *P. aeruginosa* with MIC of 3.125 µg/mL. However, hexane extract of pulp showed higher inhibitory activity against *E. coli* with least MIC (3.125 µg/mL). the antimicrobial activity of *T. bellerica* fruits was also reported in previous studies (Devi and al. 2014; Chandel and al. 2016). Study from Dharmaratne and al. (2018) reported antibacterial activity (MIC 0.25–4 mg/mL) against all strains of MRSA, MDR *Acinetobacter* spp. and MDR *P. aeruginosa* by aqueous and methanol extracts. None of the extracts exhibited activity against MDR *K. pneumoniae* (MIC > 5 mg/mL). Kumar and al. (2021) have reported good antibacterial activity with ZOI between 13 to 18 mm and MIC ranging from 6.25 to 1.56 µg/mL against tested bacteria. Aqueous and ethanolic extract of *T. bellerica* fruits showed strong antibacterial activity (MIC-4 to >32 µg/m) against imipenem and meropenem resistant *Pseudomonas aeruginosa* strains (Thirunavukkarasu and al. 2021).

Table-3: Growth inhibitory activity of various solvent extracts of *T. bellerica* fruits. Values were expressed in µg/mL.

Bacterial strains	**Hexane**			**Methanol**			**Aqueous**		
	Pulp	Seed coat	Seed	Pulp	Seed coat	Seed	Pulp	Seed coat	Seed
B. subtilis	12.5	12.5	12.5	6.25	3.125	6.25	6.25	12.5	12.5
S. aureus	25	25	25	6.25	3.125	6.25	12.5	6.25	6.25
E. coli	3.125	12.5	12.5	12.5	6.25	25	6.25	12.5	12.5

K. pneumoniae	50	50	6.25	3.125	6.25	12.5	12.5	12.5	6.25
P. aeruginosa	12.5	12.5	12.5	3.125	6.25	6.25	6.25	6.25	6.25

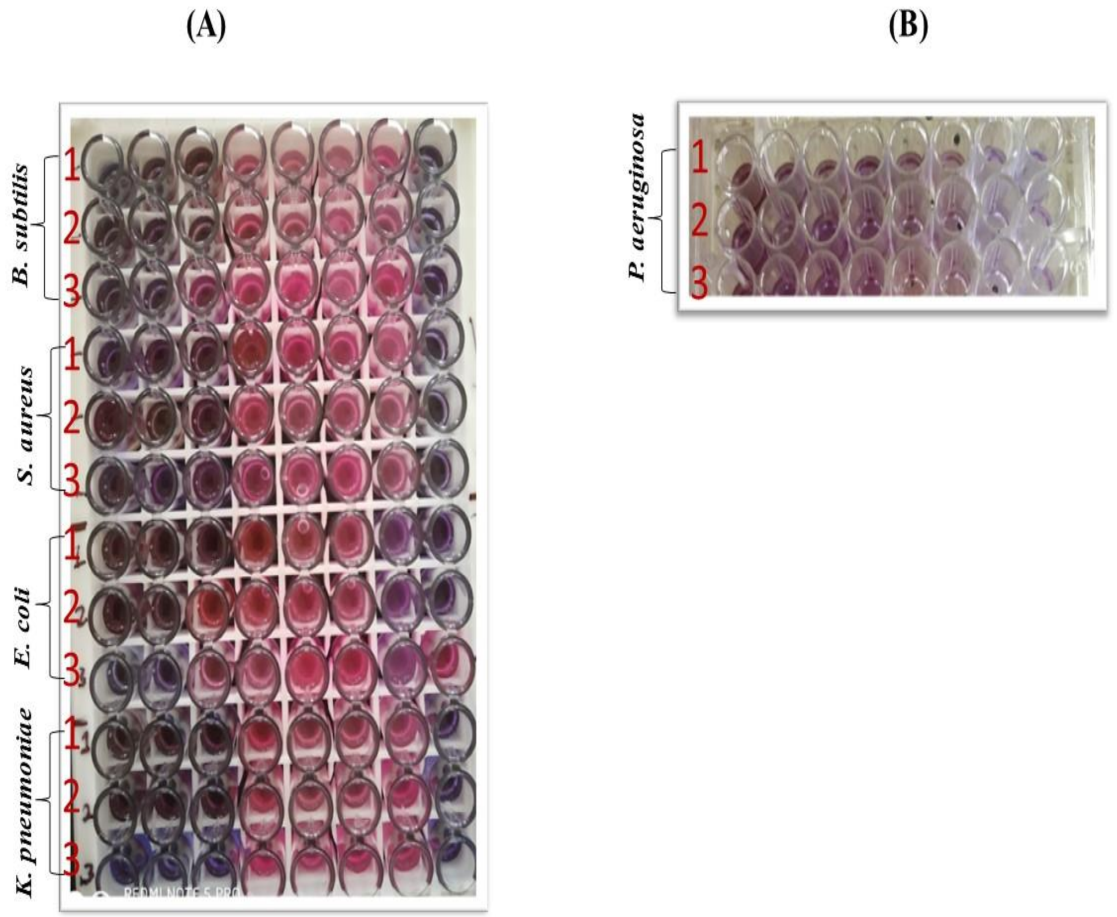


Fig. 2: Broth dilution assay performed in microtiter plates (Plate-A and Plate-B). The change in purple color of dye indicates the growth of bacteria and was read as MIC of that extract. 1-hexane, 2-methanol, and 3-aqueous extract.

In vitro antidiabetic activity of T. bellerica fruits

Antidiabetic potential of various solvent extracts of *T. bellerica* fruits was evaluated using α-amylase inhibition method. It was found that aqueous extract of seed coat and seed showed least IC_{50} value of 3.92 µg/mL and 24.86 µg/mL as compared to that of standard drug,

metformin (14.5 μg/mL). However, different solvent extracts of various parts of fruits and metformin were found to show dose dependent α-amylase inhibition activity (Fig. 3). Similar to our reports, the ethyl acetate extract of *T. bellerica* fruits exhibited comparatively better α-amylase inhibitory activity (IC_{50}-43.5 μg/mL) as compared to that of its aqueous extract (IC_{50}-74.8 μg/mL) (Gupta and al. 2020), which was further validated by *in vivo* antidiabetic study. Methanolic fruit extract of *T. bellerica* was able to inhibit α-amylase (IC_{50} value 45.20 g/mL) and α-glucosidase (IC_{50} value 175.35l g/mL) significantly than that of standard compound acarbose (Nampoothiri and al. 2011). The antidiabetic effect of *T. bellirica* fruit was attributed to presence of octyl gallate (Latha and Daisy 2013) and gallic acid (Latha and Daisy 2011).

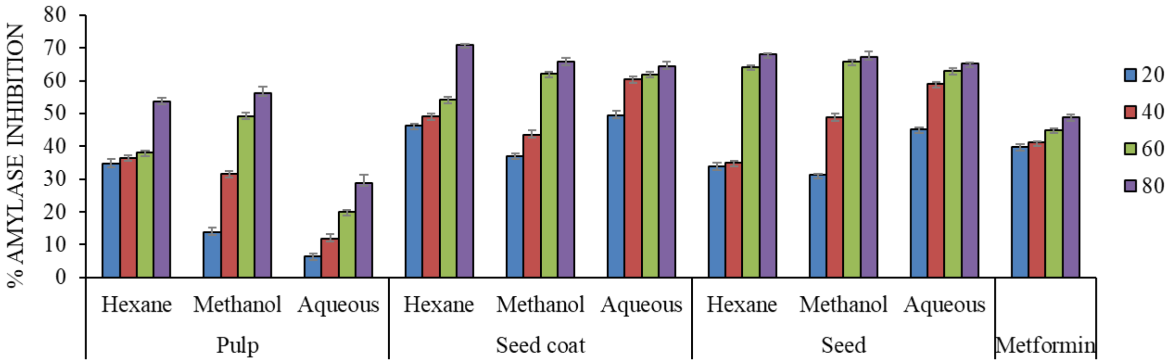


Fig. 3: Dose-dependent anti-diabetic activity shown by various extracts of different parts of *T. bellerica* fruits and standard drug, Metformin.

Conclusions:

According to the findings of the study, different portions of *T. bellerica* fruit contain significant amounts of phytochemicals, as well as antibacterial and α-amylase inhibitory action. Among all three portions, seed coat and seed of *T. bellerica* were found to be superior than that of pulp of *T. bellerica*. To comprehend the therapeutic effect of *T. bellerica* fruits, however, we still require additional substantial research to fill in the gaps in our existing body of knowledge.

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