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Evaluation of antinociceptive effect of *Terminalia arjuna* bark ethanol extract

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Abstract

Stem bark of the plant *Terminalia arjuna* were extracted in 50% ethanol to evaluate for centrally acting analgesic potential using formalin, hot plate and peripheral pharmacological actions using acetic acid induced writhing test in mice. The extract of the plant were found to have significant ($p<0.01$) analgesic activity at the oral dose of 250 & 500 mg/kg body weight, in the tested models. In hot plate test, at both dose levels (250mg/kg and 500mg/kg), *T. arjuna* extract showed significant ($p<0.001$) increased latency period than the control group. In acetic acid induced writhing test and formalin test *T. arjuna* also showed reduced number of writhes than the control group at two dose levels which are significant ($p<0.05$) compared to control. The results obtained support the use of stem bark of *T.arjuna* in painful conditions acting both centrally and peripherally.

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Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many of which based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals currently in use have been plants used by indigenous people (Balick *et al.*, 1996). Herbal medicine or phytomedicine refers to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes (Barrett *et al.*, 1999). The potential of medicinal plants can be assessed by finding new chemical entities of wide structural diversity. These new chemical substances can also serve as templates for producing more effective drugs through semi-synthetic and total synthetic procedure. According to World Health Organization (WHO), about 74% of 119 plant-derived pharmaceutical medicines or biotechnology medicines are used in modern medicine in ways that correlate directly with their traditional uses (Newman *et al.*, 2003; Barrett *et al.*, 1999). In order to promote the use of medicinal plants as potential sources of antinociceptive compounds, it is pertinent to thoroughly investigate their composition and activity and thus validate their use. Some phytochemicals produced by plants have analgesic activity allowing these plants to be studied and used for the development of new antinociceptive drugs (Uche *et al.*, 2008; Rajnarayana *et al.* 2001; Rao *et al.*, 1998). Secondary plant metabolites are largely unexplored in 'conventional' animal production systems. In the past, plant metabolites were generally considered as sources of antinociceptive factors. Recent bans and restrictions on the use of animal antibiotic growth promoters stimulated interest in bioactive secondary metabolites of plant source as alternative performance enhancers (Greathead, 2003).

Terminalia arjuna (family - Combretaceae), a large tree, is found throughout the South Asian region. It is one of the most versatile medicinal plants having a wide spectrum of biological activity (Morshed *et al.*, 2011; Ramesh *et al.*, 2004). There are very few reports regarding to its analgesic effects using various parts of this plant. Some scientists used leaves (Moulisha Biswas *et al.*, 2011) and some other scientists used crude powder (Sumita Halder *et al.*, 2009) to screen its analgesic activity.

The aim of the present work was to evaluate the analgesic assays to support the pharmacological effects and phytochemical investigation of this plant as well. Although numerous studies have shown the medicinal values of this plant, there still remains ample scope for further in depth research. So far, for the first time an attempt was taken to investigate the analgesic effect of *Terminalia arjuna* by using 50% ethanol extract of the bark. So far, there is no published report of the analgesic activity of T. arjuna bark extract with polar solvent. Accordingly, we disclose herein the antimicrobial and cytotoxic effects of the bark of *Terminalia arjuna* to further establish the scientific basis of the traditional uses of this plant.

Materials and methods

Plant materials and preparation of test sample

The barks of *Terminalia arjuna* were collected from Khamarpara, a village of Magura, Bangladesh. The plant was identified by the Bangladesh National Herbarium, Dhaka and the specimens were stored in there for the further reference (Voucher Specimen No. DACB-35235).

The stem barks of the *T. arjuna* were cut into small pieces and then water washed carefully. After washing, the fresh barks were air dried and then oven dried at 40°C temperature. The dried barks are then grinded to make powder, which were then screened to get fine powder. 1500g of barks were dried in oven and finally 500 g of fine powder was obtained. 500 g of dried bark powder were soaked in 50% ethanol. These suspensions were filtered with thin and clean cloth and then filtered by filter paper. The suspensions were evaporated by BUCHI Rota vapor R-114 [BUCHI,

Germany], connected with BUCHI water bath B-480 at 50°C. In this case, 175mbar (to remove ethanol), 72mbar (to remove water) pressure and 160rpm rotation speed were maintained constantly. Finally, small amount of liquid were evaporated from the semi-solid extracts by using a freeze-drier (HETOSICC, Heto Lab Equipment, Denmark) and 75 g of ethanol extracts were obtained.

Animals

Young male Swiss-albino mice, 4-5 weeks old (weighing 25-30 gm) were used to conduct the *in vivo* experiment. They were kept in the animal house of the Department of Pharmacy, North South University, Bangladesh and maintained at a constant room temperature of 22±5°C, 40-70% humidity conditions and the natural day-night cycle with an *ad libitum* access to food. The mice and rats had no access to food during the whole day of experiment. The influence of circadian rhythms was avoided by starting all experiments at 8.30 a.m.

Phytochemical Screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Molisch's reagent, flavonoids with the use of Mg and HCl; tannins with ferric chloride solutions and saponins with ability to produce stable foam and steroids with Libermann-Burchard reagent. Gum was tested using Molish reagent and concentrated sulphuric acid; reducing sugars with Benedict's reagent; terpenoids with chloroform and conc. sulphuric acid. These were identified by characteristic color changes using standard procedures (Ghani A., 2003).

Acute toxicity studies

The acute oral toxicity studies were performed to study the acute toxic effects and to determine minimum lethal dose of the drug extracts. Swiss albino mice and Long-Evans rat of male sex weighing 25-30 gm and 180-200 gm were used for the study. The ethanol extracts were administered orally to different groups of overnight fasted mice at the doses of 50, 100, 250, and 500 mg/kg body weight. After administration of the extracts, animals were observed continuously for the first three hours for any toxic manifestation. Thereafter, observations were made at regular intervals for 24 hrs. Further the animals were under investigation up to a period of one week (Ghosh, 1984).

Analgesic Activity

Formalin assay

The formalin test was carried out as described by previous workers (Hunskaar S. *et al.*, 1985). Four groups of mice (n = 5) were treated orally with the ethanol extract of *T. arjuna* (250 and 500 mg/kg), diclofenac (10 mg/kg) and normal saline (10 ml/kg bw). Formalin solution (0.5% v/v) was injected into the sub-plantar region of the right hind paw of the animals 30 min post treatment. The number of times paw was licked/bitten within the time frames of 0-5 min (neurogenic phase) and 15-30 min (inflammatory phase) after formalin administration was counted.

Acetic acid induced writhing method

To evaluate the analgesic effects of the plant extract, the method described by Dharmasiri *et al.* (2003) was used with slight modifications. Different groups of five mice each received orally normal saline solution (10 ml/kg) (i.e. control), diclofenac (10mg/kg), or plant extract (250 and 500mg/kg). Thirty minutes later, 0.6% acetic acid (10ml/kg) solution was injected intraperitoneally to all the animals in the different groups. The number of writhes (abdominal constrictions) occurring between 5 to 15 min after acetic acid injection was counted. A significant reduction of writhes in tested animals compared to those in the control group was considered as an antinociceptic response.

Hot- plate test method

The hot-plate test method was employed to assess the analgesic activity which was previously described by Lanhers *et al.* (1992) and modified by Mahomed and Ojewole (2004). The experimental mice were divided into four groups designated as group-I, group-II, group-III and group-IV consisting of five mice in each group for control, positive control and test sample group respectively. Each group received a particular treatment i.e. control (distilled water, 10ml/kg), positive control (Diclofenac sodium 10mg/kg) and the test sample (50% ethanol extract of 250 mg/kg & 500 mg/kg respectively). The animals were positioned on hot plate kept at a temperature of 55±0.5 °C. Reaction time was recorded when animals licked their fore or hind paws, or jumped prior to and 0, 30, 60, 120,180 and 240 min after oral administration of the samples. Percent analgesic score was calculated as (PAS) = (Tb-Ta/Tb) × 100; where, Tb= Reaction time (in second) before drug administration; Ta = Reaction time (in seconds) after drug administration.

Statistical analysis

Data from the experiments were analyzed using the Statistical Package for Social Science (SPSS) software for windows version 17 (SPSS Inc., Chicago, Illinois, USA). All the triplicate data were expressed as Mean ± SD as appropriate. Statistical analysis of the results was performed by using the One way ANOVA (analysis of variance) followed by Bonferroni post hoc and Dunnett test. The limit of significance was set at $p<0.05$.

Results

Photochemical screening

Phytochemical screening of the crude extract revealed the presence of tannins, flavonoids, saponins, gums, steroids, alkaloids, reducing sugar and terpenoids. The intensity of the component content was high in all of the tested groups except saponins and terpenoids (Table 1).

Table 1. Result of chemical group test of the 50% ethanol extract of *Terminalia arjuna*.

Plant Extract	Tanins	Flavonoids	Saponins	Gum & Carbohydrate	Steroids	Alkaloids	Reducing sugar	Terpenoids
50% ethanol	+++	+++	++	+++	+++	+++	+++	++

High = +++; Moderate = ++

Table 2. Effect of *Terminalia arjuna* ethanol extract on formalin-induced pain in mice.

Group	Control	Standard (Diclofenac)	*T. arjuna* (250 mg/kg)	*T. arjuna* (500 mg/kg)
Early phase (0-5 Min)	25.25±0.47	12.75±0.75***	20.25±1.31*	14.50±1.32***
Late phase (15-30 Min)	14.50±1.65	4.00±0.40***	7.12±0.40**	5.50±0.54***
% Inhibition				
Early phase (0-5 Min)		49.50	19.80	43.56
Late phase (15-30 Min)		72.41	50.89	62.08

Values are presented as mean ± SEM (n=7 animals per group). *P< 0.05, **P< 0.01, ***P< 0.001 vs. control

Table 3. Effect of *Terminalia arjuna* extract on acetic acid-induced writhing in mice.

Group	Number of writhing (5-15) Min	% Inhibition
Control	44.25±3.54	
Standard (Diclofenac)	18.75±0.47***	57.63
T. arjuna (250 mg/kg)	29.75±1.49**	32.76
T. arjuna (500mg/kg)	22.50±1.04***	49.15

Values are presented as mean ± SEM (n=7 animals per group). **P< 0.01, ***P< 0.001 vs. control.

Table 4. Hot- plate test for *Terminalia arjuna* ethanol extract.

	Experimental Groups			
Treatment	Control (n=5)	Positive control (n=5)	*T. arjuna* (250 mg/kg) (n=5)	*T. arjuna* (500 mg/kg) (n=5)
0 Min	12.36±1.23	9.46 ± 0.65	11.98 ±1.52	8.04 ±1.41
30 Min	10.74±0.89	12.20±0.44	13.76±1.64	10.84±1.25
60 Min	9.22 ± 0.66	13.70±0.47**	15.36±1.26*	12.46±0.95**
120 Min	8.14±0.60	15.28±0.50***	16.54±0.77***	13.32±1.02***
180 Min	6.66±0.55	6.66±0.55	17.46±0.59***	14.18±0.97***
240 Min	6.02±0.54	14.40±0.29***	11.22±0.87*	10.80±0.67***
	% Inhibition			
30 Min		28.96	14.84	34.82
60 Min		44.82	28.21	54.97
120 Min		61.52	38.06	65.07
180 Min		79.70	45.74	76.37
240 Min		52.21	6.34	34.32

Values are presented as mean±SEM (n=5 animals per group). *P<0.05, ***P*< 0.01, ****P*< 0.001 vs. control.

Acute toxicity

Acute toxicity studies show that drug is safe up to the dose of 500 mg/kg with the 50% ethanol extract of *T. arjuna* to rats and mice. No mortality was recorded in any group after 72h of administering the extract to the animals.

Formalin assay

Treatment with the ethanol extract at 250, 500 and diclofenac at 10mg/kg caused significant decrease in licking time and frequency of licking of the formalin-injected paw of mice (Table 2). The 500mg/kg dose showed the highest effect.

Acetic acid induced writhing in mice

The ethanol extract of *T. arjuna* and diclofenac induced significant decrease in the number of writhes when compared to the control (Table 3). The extract at both concentrations showed significant result but the dose 500 mg/kg showed more significant result than the 250 mg/kg dose level.

Hot-plate test

Results of hotplate test are presented in table 4 for the ethanol extract of *T. arjuna*. The extract of the plant was found to exhibit a dose dependent increase in latency time when compared with control. At 180 and 240 minutes, the percent inhibition of two different doses (250 and 500 mg/kg body weight) were 45.74% & 6.34% and 76.37% & 34.32% for *T. arjuna* respectively. The results were found to be statistically significant (p<0.001).

Discussion

From acute toxicity studies it was found that the drug is safe up to the dose of 500 mg/kg with 50% ethanol extract because it did not show any significant changes in behavior, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects

during the observation period. In future it will provide high margin of safety during formulation.

In formalin test, the pain in the early phase was due to the direct stimulation of the sensory nerve fibres by formalin while the pain in the late phase was due to inflammatory mediators, like histamine, prostaglandins, serotonin and bradykinins (Murray *et al.* 1988, Tjolsen *et al.* 1992, Dharmasiri *et al.* 2003). Formalin test is believed to be a more valid analgesic model which is better correlated with clinical pain (Tjolsen *et al.* 1992, Ghannadi *et al.* 2005). In this study, the 50% ethanol extract of *T. arjuna* caused a dose-dependent decrease in licking time and licking frequency by the mice injected with formalin signifying the analgesic effect of the extract. The extract showed significant result with both of the dose levels (250mg/kg and 500mg/kg bw) but the higher percentage of inhibition (62.08%) and significant result ($p<0.001$) was found with 500mg/kg dose level.

Acetic acid induced writhing in mice attributed visceral pain finds much attention of screening analgesic drugs (Hasan *et al.*, 2010). The ethanol extract of the *T. arjuna* plant showed significant analgesic action ($p<0.01$ and $p<0.001$) compared to the reference drug diclofenac against acetic acid induced pain in mice at two dose levels i.e. 250 & 500 mg/kg bw which is consistent with some other investigators though in that case they used different parts and extracts of *T. arjuna* (Moulisha Biswas *et al.*, 2011; Sumita Halder *et al.,* 2009). Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipid (Ahmed *et al.*, 2006) via cyclooxygenase (COX), and prostaglandin biosynthesis (Duarte *et al.*, 1988). In other words, the acetic acid induced writhing has been associated with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products (Derardt *et al.*, 1980). The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Zakaria *et al.* 2008). The acetic acid induced writhing method was found effective to evaluate peripherally active analgesics. The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (Duarte *et al.*, 1988; Ferdous *et al.*, 2008). The significant pain reduction of both the plant extracts might be due to the presence of analgesic principles acting with the prostaglandin pathways.

The extracts of the plants and diclofenac sodium (10 mg/kg) also presented a longer latency time than the control group in the hot plate test in a dose related manner. At 180 minutes, 250 mg/kg and 500mg/kg, p.o. administration of the *T. arjuna* plant extract, the percent inhibition was found 45.74% and 76.37% respectively which were statistically significant ($p<0.001$). Other scientists also found analgesic activity with the leaf extract by using this method (Moulisha Biswas *et al.,* 2011). The hot plat method is considered to be selective for the drugs acting centrally. The hot plat test measures the complex response to a non-inflammatory, acute nociceptive input and is one of the models normally used for studying central nociceptive activity (Sabina *et al.*, 2009). It is an established fact that any agent that causes a prolongation of the hot plate latency using this test must be acting centrally (Ibironke and Ajiboye, 2007). Therefore, the ethanol extract of the plant must have a central activity. The plant extract of *T. arjuna* exhibited both types of pain inhibition. The analgesic effect of the plants in both models suggests that they have been acting through central and peripheral mechanism (Sabina *et al.*, 2009).

Preliminary qualitative phytochemical screening reveals the presence of alkaloids, carbohydrates, tannins, gums, terpenoids & flavonoids in *T. arjuna*. Therefore, it is assumed that these compounds may be responsible for the observed analgesic activity. Flavonoids were reported to have a role in analgesic

activity primarily by targeting prostaglandins (Rajnarayana *et al.* 2001; Rao *et al.*, 1998). There are also reports on the role of tannins in anti-nociceptive activity (Vanu *et al.*, 2006). Besides alkaloids are well known for their ability to inhibit pain perception (Uche *et al.*, 2008).

In conclusion, since the plant extract reduced significantly the reduced the number of writhes in formalin, acetic acid induced writhing models and hot plat test, the 50% ethanol extract of the stem bark of *T. arjuna* exhibited strong analgesic activities. Again, no mortality was recorded in the acute toxicity test; it showed that the plant is safe for use. The study has thus provided some justification for the folkloric use of the plant in several communities for conditions such as stomachache, pain and inflammations.

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