ACUTE TOXICITY AND ANTIINOCICEPTIVE ACTIVITY OF SAPONINS RICH FRACTION OF DIOCOREA DELTOIDEA (WALL)

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ABSTRACT

OBJECTIVE: To evaluate the saponins rich fraction of Dioscorea deltoidea (D. deltoidea) for possible antinociceptive activity.

METHODS: Saponins were extracted from the crude methanolic extract of D. deltoidea (Wall). Presence of saponins was confirmed through phytochemical screenings. Acute toxicity test was performed to determine safe dose range of the saponins rich fraction (n-butanol fraction) using balb C Mice. The saponins rich fraction was assessed for possible antinociceptive activity using acetic acid induced writhing method and hot plate method. Data was analyzed using Graph Pad Prism.

RESULTS: The results showed that crude saponin rich fraction was safe up to test dose of 1000 mg/kg administered orally. In acetic acid induced writhing method, its test samples in doses of 10 and 50 mg/kg showed respectively 75.31% and 85.07% inhibition of writhing, while diclofenac sodium showed 74.4% inhibition of writhing. Percent inhibition of latency time of test samples increased from 87.87% to 133.6% in test doses of 10 mg/kg to 100 mg/kg dose, respectively, while Tramadol showed latency time of 85.12% within 30 minutes of its administration.

CONCLUSION: Saponins rich n-butanol fraction of Dioscorea deltoidea (D.deltoidea) showed significant antinociceptive activity.

KEY WORDS: Diclofenac Sodium (MeSH); Analgesics (MeSH); Dioscorea deltoidea (Non-MeSH); Writting (Non-MeSH); Tramadol (MeSH); N-butanol (MeSH); Ethyl-acetate (Non-MeSH); chloroform (MeSH); N-hexane (Non-MeSH); Acetic Acid (MeSH).

INTRODUCTION

Pain is an unpleasant emotional and sensory experience related to potential or actual tissue damage.1 Medicinal plants are constantly used since creature of human beings for the management of various diseases including pain. Opiates and non-steroidal anti inflammatory drugs (NSAIDs) are mostly used analgesic drugs, which may not be helpful in all cases, because of their adverse effects and poor pain management. Opiates cause tolerance, addiction and physical dependence.2 NSAIDs are associated with gastrointestinal disturbances like stomach and or duodenal ulceration. Morphone is associated with tolerance, hypotension and drug dependence.3 Thus, search for a safe and effective analgesic is the need of the time. Plants of medicinal importance are in use for therapeutic purposes since many years. Many of these plants had been used for management of pain with acceptable safety profile.4

Family Dioscoreaceae consists of 600 species that are distributed throughout the world. Mostly they are found in the tropical regions of the world.5 Dioscorea deltoidea belongs to family Dioscoreaceae that produces rhizomes or tubers, which have rich economical as well as medicinal significance. D. deltoidea is one of the rare species of Pakistan.6 It is a perennial climber and hairless plant. Locally, it is named as Qanis, Varahikand, Singly-mingly, Kildri. Its rhizomes are arranged alternately and ligneous irregular. They are mostly in ginger type shape. Leaves are mostly simple and pointed, often heart shaped. In Pakistan, this species is mainly present in Kaghan Valley, Swat, Chitral, Shonala, Dir, Hazara, Galis, Kurrum, Kashmir and in Murree regions.7 In Pakistan, its germination starts after the melting of snow in months of April-May, while its flowering period is June-July. It gives fruits in August-September.8

The chemical constituents found in D. deltoidea are 25-D-spirostan-3,5 diene, B-sitosterol, dioscorine, smilagenone, campastrol, diosgenin, stigmasterol and dicosin. Main reported constituents are ascorbic acid, aluminum, riboflavin, ash, chromium, calcium, niacin, cobalt, magnesium, beta-carotene, manganese, iron, phosphorus, selenium, protein, potassium, sodium, silicon, thiamine, zinc and tin. These chemical ingredients make this plant a manufacturing plant.9

Dioscorea deltoidea is used as wormicidal substance in children. Its rhizomes are used in treating biliary colic.10 The tuberous part of the plant is...
### TABLE I: RESULTS OF ACUTE TOXICITY STUDY IN MICE FOR CRUDE SAPONIN RICH FRACTION OF DIOSCOREA DELTOIDEA

| Phases | Groups            | Dose (mg/kg) | % Lethality | % Survivors |
|--------|-------------------|--------------|-------------|-------------|
|        | Normal saline (Nil)* |              | 0           | 100         |
| Phase I| Group I           | Test substance 0.1 | 0          | 100         |
|        | Group II          | Test substance 1 | 0          | 100         |
|        | Group III         | Test substance 10 | 0         | 100         |
|        | Group IV          | Test substance 100 | 0        | 100         |
| Phase II| Group VI        | Test substance 500 | 0       | 100         |
|        | Group VII         | Test substance 1000 | 0      | 100         |

*Normal saline is only used as a vehicle

### TABLE II: RESULTS OF CRUDE SAPONINS OF DIOSCOREA DELTOIDEA ON VARIOUS ORGANS DURING ACUTE TOXICITY STUDY

| Symptoms                  | Intensity/Remarks | Normal Saline | Test Substance doses (mg/kg) |
|---------------------------|-------------------|---------------|-----------------------------|
|                           |                   | 0.1 | 10 | 100 | 500 | 1000 |
| Increase motor activity   | No                | No  | No | No  | No  | No  |
| Tremors                   | No                | No  | No | No  | No  | No  |
| Clonic convulsion         | No                | No  | No | No  | No  | No  |
| Tonic extensor            | No                | No  | No | No  | No  | No  |
| Straub's reaction         | No                | No  | No | +++ | ++++ | ++++ |
| Piloerection              | No                | +   | ++ | +++ | ++++ | ++++ |
| Catatonia                 | No                | No  | No | No  | No  | No  |
| Opisthotonos              | No                | No  | No | No  | No  | No  |
| Hyperesthesia             | No                | No  | No | No  | No  | No  |
| Loss of righting reflex   | No                | No  | No | No  | No  | No  |
| Decreased motor activity  | No                | No  | No | No  | No  | No  |
| Ataxia                    | No                | No  | No | No  | No  | No  |
| Sedation                  | No                | No  | No | +++ | ++++ | ++++ |
| Hypnosis                  | No                | No  | No | No  | No  | No  |
| Analgesia                 | No                | No  | +  | +++ | ++++ | ++++ |
| Anesthesia                | No                | No  | No | No  | No  | No  |
| Arching and rolling       | No                | No  | No | No  | No  | No  |
| Ptosis                    | No                | No  | No | +   | ++++ | ++++ |
| Larcimation               | No                | No  | No | No  | No  | No  |
| Exophthalmos              | No                | No  | No | No  | No  | No  |
| Salivation                | Watery            | No  | No | No  | No  | No  |
|                          | Viscid            | No  | No | No  | No  | No  |
| Diarrhea                  | No                | No  | No | No  | No  | No  |
| Shivering                 | No                | No  | +  | +++ | ++++ | ++++ |
| Respiration               | Depression        | No  | No | No  | No  | No  |
|                          | Stimulation       | No  | No | No  | +   | ++++ | ++++ |
|                          | Failure           | No  | No | No  | No  | No  | No  |
|                          | Blushing          | No  | No | No  | No  | No  |
|                          | Flushing          | No  | No | No  | No  | No  |
|                          | Cyanosis          | No  | No | No  | No  | No  |

### METHODS

The study was performed in Department of Pharmacology, Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Khyber Pakhtunkhwa, Pakistan. Ethical Board of the Khyber Medical University, Peshawar accorded approval of the Study protocols via approval No. DIR/KMUJ-EB/AT/000365.

**Drugs and chemicals:** Analytical grade chemicals were used. solvents and drugs used in experimental work such as methanol 80%, n-butanol, chloroform, n-hexane, ethyl acetate, distilled water, tramadol, 0.7% acetic acid, diclofenac sodium and 0.9% normal saline.

**Animals:** Swiss albino mice of either sex having weight in range of 25-40 g were used in experiments. These mice were purchased from the animal house of Department of Pharmacy, University of Peshawar, KP, Pakistan. The mice were aclitimized on standard laboratory conditions (25±2°C; 12 hours light – 12 hours dark cycles). The animals were fed with standard food and water.

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taken orally to cure dysentery, hemorrhoids and abdominal pain.1,12

The solution of Dioscorea deltoidea from rhizomes are used to cure ailments of central nervous system (CNS), orthopedic disorders, dermatitis, metabolic disorders, cardiovascular system, autoimmune diseases, and in oncology. The rhizomes of Dioscorea deltoidea are used to treat various diseases like digestive disorders, diarrhea, sore throat, irritability, burns, abdominal pain, anemia and wounds. Steroidal glycosides of D. deltoidea possess hypo-cholesterolemic, fungicide, antimicrobial, hemolytic, antitumor and biological activities.7,13 Diosgenin is a precursor to synthesize progesterone which is used in oral contraceptive pills.14 The tubers of D. deltoidea contain phytoconstituents like alkaloids, steroids, fats and fixed oil, flavonoids, tannins, proteins, carbohydrates and saponins.8,15 Saponins have been reported to possess a wide range of biological activities like antiulcer, antiviral, chemo preventive, cytotoxic, diuretic, analgesic and antiallergic.15 In general, saponins show analgesic activity. It has been previously tested for analgesic activity using animal models. Saponins inhibit writhing in mice and have showed analgesic activity.17 More, it has been reported that Dioscorea deltoidea has anti-inflammatory and antimicrobial activity.16 Based on the phytochemicals and antiinflammatotory model in animals, we carried out current study to screen the saponins rich fraction of Dioscorea deltoidea for possible antinociceptive activity.
After completion of the experimental protocols, the animals were disposed off as per procedure adopted by Safety and Bioethics Committee of the Institute of Basic Medical Sciences, Khyber Medical University.

Collection and identification of plant materials: The fresh rhizomes of Dioscorea deltoidea were collected from Sheringal valley, Dir (Upper), Khyber Pakhtunkhwa, Pakistan. The plant was collected in the month of July, which is its peak collection time. The plant was identified by Professor Dr. Jehandar Shah ex-vice chancellor and plant taxonomist, University of Malakand, Pakistan. A voucher specimen Dd-2014 has been submitted to Department of Pharmacology, Khyber Medical University, Peshawar.

Isolation and extraction of plant materials: The fresh rhizomes of Dioscorea deltoidea were collected, washed and then dried in the shade for three weeks. The rhizomes were cut down into small pieces and then converted into fine powder with help of a mechanical grinder. The powdered material (2.0 kg) was soaked for one week in commercial grade methanol (80%) on room temperature with continuous shaking. The menstruum was then filtered through muslin cloth. This whole process was repeated three times. The different filtrates were then combined and filtered through Whatmann filter paper (No 1). The filtrates were evaporated in a rotary evaporator on 40ºC till a semisolid material (2.0 kg) was obtained.21

Fractionation and phytochemical screenings: The crude methanolic extract (85.0g) was suspended in distilled water. It was then successively fractionated with n-hexane, chloroform, ethyl-acetate and n-butanol. Each fraction was evaporated to dryness at 40ºC using a rotary evaporator till it respectively yielded, n-hexane (0.607 g), chloroform (6.42 g), ethyl-acetate (5.39 g), n-butanol (2.8 g) and residual aqueous fraction (12.15 g). The fractions were tested for the presence of saponins using frothing test and emulsification test.22 Frothing test was performed by adding different filtrates in 10ml of distilled water one by one. The mixture is shaken vigorously for 7-10 minutes. The formation of froth on the upper surface of mixture confirms the presence of saponins. Emulsification test was performed by the addition of 2-3 drops of olive oil in solution having froth formation. The solution was forcefully shaken for 2 minutes. The formation of uniform emulsion confirmed the existence of saponins.22 Each fraction was stored in airtight bottle at room temperature. The saponin-rich fraction was diluted in water for injection for in-vivo studies.21

Acute toxicity assay: It was performed on Swiss albino mice. Mice of either sex, having weight 25-40 g, were used in the experiments. It was performed in two phases. Exponential doses of saponins rich fraction (n-butanol) in test doses of 0.1, 1, 10, 100 mg/kg were administered p.o in phase I. While in phase II, 500 and 1000 mg/kg were administered orally.21 Death toll was recorded in 24 hours. Morbidity score was also performed to know about its possible unwanted effects.

Antinociceptive activity: Antinociceptive activity was evaluated by the following two methods.

Acetic acid-induced writhing method: Swiss albino mice of either sex having weight 25-40 g were used in this method. Animals were divided into six groups having four animals in each group. Pain sensation was induced by administrating 0.7% acetic acid (i.p). Abdominal constriction (writhing) is an indication of pain due to release of local prostaglandins. Normal saline was administered to group I, which served as negative control. Diclofenac sodium 50 mg/kg was administered to group II, which served as a standard group. Saponin rich fraction (n-butanol fraction) in the test doses of 1, 5, 10, 50 mg/kg were respectively administered to test groups III, IV, V, VI (i.p). After 30 minutes, 0.7% acetic acid was administered (i.p) to each mouse to induce abdominal constrictions. After 5

| TABLE III: EFFECT OF CRUDE SAPONIN EXTRACT OF DIOSCOREA DELTOIDEA ON ACETIC ACID INDUCED WRITHING MICE |
|---------------------------------|------|----------------------------|
| Treatment                      | Groups | Dose (mg/kg) | Number of % inhibition of before and after drug administration  |
|--------------------------------|------|----------------------------|
| **Negative control group**     | Group I | Normal saline (Nil) | 49 ± 6.5 | --- |
| Test groups                    | Group II | Test substance 1 | 37.5 ± 2.12 | 23.5 |
|                               | Group III | Test substance 5 | 21.5 ± 6.36 | 56.12 |
|                               | Group IV | Test substance 10 | 12 ± 5.65 | 75.51 |
|                               | Group V | Test substance 50 | 7.1 ± 4.1 | 85.71 |
| **Standard group**             | Group VI | Diclofenac sodium 50 | 12.5 ± 3.53 | 74.4 |

| TABLE IV: EFFECT OF CRUDE SAPONIN EXTRACT OF DIOSCOREA DELTOIDEA ON LATENCY TIME IN HOT PLATE TEST |
|-------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Treatment Group I                                           | Group I | Normal saline (Nil) | 12.75 ± 5.72 | 11.85 ± 2.19 | 12.85 ± 6.29 | 11.15 ± 1.66 | 12.8 ± 0.84 |
| **Test groups**                                             | Group II | Test substance 10 | 12.2 ± 5.37 | 27.8 ± 0.28* | 23.1 ± 3.39* | 20.75 ± 0.69* | 17.65 ± 2.33* |
| Group III | Test substance 50 | 11.67 ± 5.4 | 29.65 ± 1.48* | 29.1 ± 0.98* | 26.55 ± 0.35* | 22.6 ± 1.41* |
| Group IV | Test substance 100 | 13.5 ± 2.00 | 36.1 ± 1.53* | 35.1 ± 0.84* | 36.5 ± 4.66* | 26.7 ± 4.52* |
| **Standard group**                                          | Group V | Tramadol 30 | 13.4 ± 1.23 | 27.3 ± 3.11* | 27.05 ± 2.47* | 26.1 ± 1.69* | 22.5 ± 6.92* |

All values are mean ± SD, n=4 *P < 0.05 versus Control Group.
minutes of acetic acid administration, writhings were counted for 10 minutes. Each mouse was observed separately for accurate measuring. The numbers of writhing in the test group was compared with standard control group. The percent inhibition of writhing was calculated to determine analgesic activity using following formula:

\[
\% \text{ Inhibition of writhing} = \frac{\text{Writing in Control Group} - \text{writing in test group}}{\text{writing in test group}} \times 100
\]

**Phytochemical Screening:**

Phytochemical test revealed that saponins were rich in n-butanol fraction and residual aqueous fractions as it produced significant frothing and uniform emulsion upon addition of olive oil.

**Effects of acute toxicity activity:** The crude saponin rich n-butanol fraction was safe up to 1000 mg/kg, and there were no mortalities found (Table I). There were no gross behavioral changes for 24 hours study while sedation, prostration, increased respiration, piloerection, strabismus’ reaction and shivering were observed on higher doses as shown in Table II. According to the results, the fraction was safe up to 1000 mg/kg and can be used for further studies. However, sedation and analgesia in this phase guided us to conclude that the test samples have possible analgesic activity through involvement of CNS.

**Analgesic activity:** Results for possible analgesic activity are as under:

**Acetic acid induced writhing method:** According to the results, test samples on 10 and 50 mg/kg showed maximum inhibition of writhings as compared to negative control group. Test substance in 10 mg/kg and 50 mg/kg showed 75.51% and 85.71% inhibition of writhing, while diclofenac sodium showed 74.4% inhibition of writhing. This implies that test substance is more potent as compared to our standard drug as shown in Table III.

**Hot plate method:** Eddy’s hot plate method was used to determine analgesia. Swiss albino mice of either sex were used having weight 25-40 g. Animals were divided into five groups, four animals in each group. Hot plate was maintained on 55°C before the start of the experiments. Hot plate generates electrical heat that causes pain. The indication of animal’s response to pain induced by heat was licking of the hind paw and coming out of the beaker. Group I received normal saline, which served as negative control. Group II received tramadol 30 mg/kg, which served as positive control. Saponin rich fraction (n-butanol) in the test doses of 10, 50, 100 mg/kg were administered to group III, IV, V respectively through intraperitoneal route. Readings were taken on 0, 30, 60, 90 and 120-min intervals using a hot plate. The percent latency time was calculated using the following formula:

\[
\% \text{Analgesia} = \frac{(\text{Cut off time} - \text{Control latency time})}{\text{Test latency} - \text{Control latency time}} \times 100
\]

**Statistical analysis:** For LD_{50}, percent mortality was plotted versus test dose administered. Number of writhing were noted and % inhibition of writhing was determined versus respective standard drug. Latency time was calculated and plotted amongst different groups using tramadol as standard drug. Data was plotted and analyzed using Graph Pad Prism 6. ANOVA was used to determine the significance of test samples versus positive control at 95% CI with p<0.05.

**RESULTS**

**Phytochemical Screening:**

The main focus of our study is to confirm the possible antinociceptive activity of crude saponins rich fraction of Dioscorea deltoidea. This was accomplished by two different methods. The acetic acid-induced writhing method is due to the involvement of peripheral nervous system while thermal nociception models that is hot plate method is due to the involvement of central nervous system mechanism for possible analgesia. Arachidonic acid has an important role in pain and inflammation. It causes the release of inflammatory mediators like prostaglandins and cytokines through cyclo-oxidase and lipooxygenase pathways as prostaglandins mediate pain. Acetic acid causes pain sensation by the release of inflammatory mediators. Phospholipids are present in tissues that cause the release of arachidonic acid which in turn causes the synthesis of prostaglandins through cyclo-oxidase pathway. These prostaglandins stimulate the nociceptive neurons with initiation of pain sensation. The main prostaglandins that are involved in pain are PGF2 and PGE2 that are present in peritoneal fluids. The increase in prostaglandins level causes pain by increasing the capillary permeability. The writhing/abdominal constriction method is widely used to assess possible analgesic activity of a test sample that acts through the peripheral pathway. Literature suggests that any substance which inhibits the writhing/abdominal constrictions will have possible analgesic action by inhibiting the release of prostaglandins.

NSAIDs inhibit the cyclo-oxygenase pathway and subsequently inhibit the release of prostaglandins. Hence, the analgesic activity of NSAIDs confirm the association of peripheral mechanism in pain reduction. That is why NSAIDs are used as positive control in writhing model. Thus, saponins rich samples follow the peripheral pathway for inhibition of the release of prostaglandins. Maximum analgesic action was observed with 10 mg/kg and 50 mg/kg that respectively decreased the writhings by 75.51% and 85.71%. While diclofenac sodium decreased writhings by 74.4%.

Thermal nociception model such as hot plate method also predicts the possible analgesic activity through involvement of central nervous system. Thermal nociceptor neurons are excited by thermal noxious stimulus in the skin or
in visceral organs. This is best explained as thermal stimulus induces pain due to which animal withdraws its hind paw. Thus, our results suggest that test sample in dose of 50 mg/kg and 100 mg/kg showed more latency time as compared to effects produced by tramadol within 30 minutes of its administration. The difference in latency time could be explained by the difference in the metabolic rate of each drug, or maybe there is the difference in potency of each drug. Moreover, crude saponins rich fraction of D. deltoidea showed maximum analgesic effect within 30 minutes of administration, which sustained for 120 minutes. Literature suggests that the Dioscorea species show analgesic activity. Thus, it is deduced that analgesic effects of crude saponin rich fraction of D. deltoidea involve both peripheral and central mechanisms.

CONCLUSION

Our current results confirm that n-butanol fraction of Dioscorea deltoidea has analgesic activity through involvement of both peripheral as well as central nervous system nociceptive inhibitory mechanisms. Subsequent extraction and pharmacological screening of the phytochemicals of saponins may help in development of new analgesic drugs from the saponin rich fraction of the Dioscorea deltoidea.

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AUTHORS’ CONTRIBUTIONS

Following authors have made substantial contributions to the manuscript as under:

NA: Conception and study design, analysis and interpretation of data, drafting the manuscript, critical review, final approval of the version to be published.

N, MN: Acquisition of data, drafting the manuscript, final approval of the version to be published.

ZS, US & BS: Acquisition, analysis and interpretation of data, drafting the manuscript, final approval of the version to be published.

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Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

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