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Antioxidant activity & wound healing activities extract of *Xanthium strumarium* leaves

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Abstract
Wound healing activity of ethanolic extract of leaves of *Xanthium strumarium* was studied by Chorioallantoic membrane (CAM) model (*In vitro*) in 09-day old embryonated chicken eggs. The extract also promoted angiogenesis as evidenced by the CAM model. The result suggested that ethanolic extract of *Xanthium strumarium* possess significant wound healing potential in the normal wound. The preliminary phytochemical screening of the successive extracts of selected plant species viz., methanol aqueous extract confirmed the presence of carbohydrates, glycosides, alkaloids, phenolics and tannins, saponins, flavonoids. The phytochemical findings of the study confirm the presence of plant phenolics, flavonoids and other secondary metabolites which are currently of growing interest owing to their functional properties in promoting human health. Flavonoids and other plant phenolics act as remedies in the treatment of stress-related ailments and as dressings for wounds, cuts, rheumatism etc.In present investigation, objective of the study was to extract the *Xanthium strumarium* L. leaves and evaluate its phytochemical and wound healing potential along with antioxidant property. The ethanolic extract of *Xanthium strumarium* leaves had shown activity from slight to mark which was dose dependent high dose of extract (400mg) showed the marked increases in blood vessels.

Keywords: chorioallantoic membrane model, wound healing, antioxidant activity, phytochemical screening, *Xanthium strumarium*

Introduction
A wound may be defined as a break in the epithelial integrity of skin or loss of cellular anatomic or functional continuity of living tissue. Wounds are a major cause of physical disabilities. When skin is torn, cut, or punctured it is term as an open wound and when blunt force trauma causes a contusion, it is called a closed wound, whereas burn wounds are caused by fire, radiation, chemicals, heat, sunlight or electricity. Normal wound healing begins immediately after the tissue is injured. In undamaged skin, the epidermis (surface layer) and dermis (deeper layer) form a protective barrier against the external environment. When the barrier is broken, a regulated sequence of biochemical events is set into motion to repair the damage. Wound healing involves different phases including hemostasis, inflammation, fibrogenesis, granulation, wound construction, neo-vascularization and epithelization. The various natural and synthetic drugs are available for the treatments of wounds and are commonly known as wound healing agents.
The use of the herbal extract in place of crude herbs started with the aim to control quality and precise dosage for better results. The plant extracts being more efficacious, free from undesirable side effects compared to their pure active principle revalidated the therapeutic benefits of herbs due to the totality of constituents rather than the single molecule.
Xanthium strumarium common medicinal plant belongs to Asteraceae. The plant occurs all over Pakistan, India, China, and Eurasia and also in America. The local name of *Xanthium strumarium* is Common Cocklebur and Chota dhatura. The 20,000 species of its 950 genera are found worldwide as herbs, shrubs, trees and climbers. It is commonly found as a weed in roadsides, rice fields, and hedges throughout the tropical parts of India. *Xanthium strumarium* is an annual herb, up to 1 m in height. *Xanthium strumarium* has stout stems, green, brownish or reddish-brown, often red-spotted that are rough and hairy. These medicinal properties are due to the presence of chemical constituents such as steroids, alkaloids, flavonoids, triterpenoids, terpenoids, tannins, saponins, quinone, fatty acid, coumarin, protein, sugar and gum. The extract of *Xanthium strumarium* exhibited effect antibacterial, antitumor, antitussive, antifungal, anti-inflammatory, antinociceptive, hypoglycaemic, antimitotic, antioxidant, antitrypanosomal, CNS depressant activity, diuretic effects, and contact dermatitis, insecticidal and herbicidal activities.
Although the local traditional healers have ethnomedical knowledge on the value of this plant, there have been no biological studies on the wound healing activity of this plant. Hence, the present study was undertaken to evaluate the wound healing activity of the methanolic extract of the leaves of this plant by the CAM model.

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defences which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases including cancer. A small quantity of the extract was dissolved in distilled water and filtered. The filtrate was tested to detect the presence of various phytochemical constituents in the sample.

Material and Methods

Collection and Authentication of Plant Material

The leaves of *Xanthium strumarium* L. (Asteraceae) were collected from medicinal plants garden School of Pharmacy, Hapur, Uttar Pradesh, India in July 2021 from healthy plants. Herbarium of the plant material was prepared and authenticated by Dr. (Mrs.) Sunita Garg (Chief scientist, raw materials herbarium and museum, NISCAIR, New Delhi, India) having voucher specimen no. NISCAIR/RHMD/Consult/2021/3858-59 dated 18 Aug. 2021.

Chemicals

Chloral hydrate, Fehling solution A & B, picric acid, and sodium hydroxide nitric acid, α-napthol, chloroform, petroleum ether (40-60°C), toluene, ethanol, methanol, ethyl acetate, hydrochloric acid, sulphuric acid, lead acetate, perchloric acid, potassium bismuth iodide, potassium mercuric iodide, potassium iodide, sodium bicarbonate, sodium carbonate, and glacial acetic acid; antimony dichloride, ferric chloride, and sodium nitroprusside; millon's reagent, and ninhydrin reagent. All the other chemicals and reagents used were of analytical grade.

Methods of Extraction

The fresh leaves were subjected to size reduction with the help of stainless-steel grinder and collect the fine power of the leaves. Extraction was done with hot Soxhlet extraction process using ethanol as solvent then the extracts were concentrated to dryness with the help of water bath and finally air dried. The obtained dried extracts of *Xanthium strumarium* L. leaves were weighed and extractive value was calculated. It was kept in an air tight container and stored in a desiccator and used for investigation of their potential.

Fig 1: *Xanthium strumarium* extraction process

Phytochemical screening

Screening of the extract was carried out by standard method**.** Phytochemical screening of the methanolic extract of *Xanthium strumarium* revealed the presence of alkaloid by Wagner's and Dragendorff's test, steroid by Salkowski's and Lieberman Burchardt's test, triterpenes by Salkowski's and Lieberman Borchardt's test, carbohydrate by Molisch's test and Fehling's test, proteins and amino acids by Biuret test.

Test for Alkaloids
Small amount of extract mixed with few ml of dilute hydrochloric acid. Shaken well and filtered. Following tests were performed with the obtained filtrate.

Dragendorff's test
A few drops of Dragendorff's reagent (potassium bismuth iodide solution) was added to 2-3ml of filtrate. Orange red precipitate indicates the presence of alkaloids.

Test for Flavonoids
Ammonia test
A few milligrams of the extract was dissolved in water and filtered. Filter paper strip was dipped in the filtrate and ammoniated. Yellow colouration of the filter paper strip indicates the presence of flavonoids.

Test for glycosides
Legal's test
1ml of pyridine and 1ml of sodium nitroprusside was added to 1ml of extract. Pink to red colour indicates the presence of glycosides.

Test for steroids and sterols
Liebermann- Burchard reaction
2ml of extract was mixed with chloroform. To that mixture added 1-2ml of acetic anhydride and 2drops of concentrated sulphuric acid along the sides of the test tube. The solution becomes red, then blue and finally bluish green colour.

Test for phenols
Ferric chloride test
1ml of the alcoholic solution of the extract was added to 2ml of distilled water followed by few drops of 10% ferric chloride. Formation of blue or green colour indicates the presence of phenols.

Test for tannins
Lead acetate test
A few drop of lead acetate was added to 5ml of aqueous extract. Formation of yellow or red colour precipitate indicates the presence of tannins.

Test for Saponins
Foam Test
1ml of test sample was diluted with 20ml of distilled water and shaken it in a graduated cylinder for 3minutes. Foam of 1cm after 10min indicates the presence of saponins.

Test for proteins and amino acids
Biuret test
3ml of test solution was added to 4% sodium hydroxide and few drops of 1% copper sulphate solution. Formation of violet colour indicates the presence of proteins.

Test for carbohydrates
Molisch's test
Few drops of Molisch's reagent were added to 2-3ml of filtrate, followed by addition of concentrated sulphuric acid along the sides of the test tube. Formation of violet colour ring at the junction of two liquids indicates the presence of carbohydrates

Table 1: Phytochemical screening of *Xanthium strumarium* leaves extract

S. No.	Chemical Test	Ethanolic extract
1.	Alkaloids	+
2.	Carbohydrates	+
3.	Glycosides	+
5.	Steroids	_
6.	Saponins	+
7.	Flavonoids	+
8.	Free amino acids	_
9.	Phenolics& Tannins	+
10.	Starch	_

Present (+), Absent (-)

***In-Vitro* Wound Healing Model**

Chorioallantoic membrane (CAM) model was used *to* evaluate wound healing potency. In this method, embryonated chicken eggs (09 days old) were selected and a small window (1 cm^2) was made in the shell. Through the window, a sterile disc of methyl cellulose treated with 400 or 600 µg of ethanolic extract of *Xanthium strumarium* leaves was placed inside triplicate sets of the egg at the junction of two blood vessels. The window was resealed and the eggs were incubated at 37°C in a well-humidified chamber for 72 hrs. The window was then opened and the growth of new capillary blood vessels was observed and finally compared with the control eggs containing sterile discs without any extract of the plant.

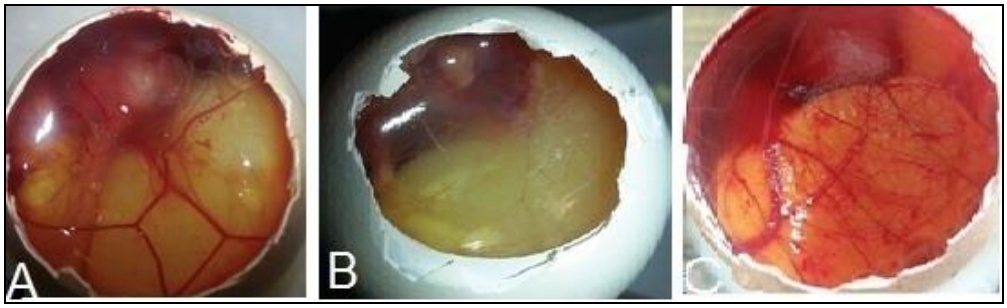

Fig 2: Angiogenesis in 09 days old chicken egg after treatment with ethanolic extract of Xanthium *strumarium*: (A)-Control containing methyl cellulose disc without plant extract, (B) Treatment containing methyl cellulose disc impregnated with 400µgethanolic extract of *Xanthium strumarium* leaves and (C) Treatment containing methyl cellulose disc impregnated with 600µg ethanolic extract of *Xanthium strumarium* leaves

The methanolic extract of *Xanthium strumarium* leaves had shown activity from slight to mark which was dose dependent high dose of the extract (600µg) showed marked increases in blood vessels. Angiogenesis plays a crucial role in wound healing by forming new blood vessels from preexisting vessels by invading the wound clot and organizing them into a microvascular network through the granulation of tissue. Angiogenesis is an essential component of wound healing. Vessel growth is controlled by the local actions of chemical mediators, the extracellular matrix, metabolic gradients, and physical forces. Manipulation of some of these factors can improve healing in experimental wounds. During wound healing, angiogenic capillary sprouts invade the fibrin/fibronectin-rich wound clot and within a few days organize into a microvascular network throughout the granulation tissue. As collagen accumulates in the granulation tissue to produce a scar, the density of blood vessels diminishes.

Antioxidant Activity

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defences which counteract the harmful effects of free radicals and other oxidants. Free radicals are responsible for causing a large number of diseases including cancer. Various methods are used to investigate the antioxidant property of samples (diets, plant extracts, commercial antioxidants etc.) like DPPH scavenging activity, Trolox equivalent antioxidant capacity (TEAC) method or ABTS radical cation decolorization assay, Peroxynitrite radical scavenging activity, and DPPH scavenging activity etc. (Kannula & Carpo 2004).

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Assay

The antioxidant activity of the methanol extracts of leaves of *X. strumarium*, mixture of leaves of were assessed on the basis of the radical scavenging effect of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The diluted working solutions of the test plant extracts were prepared in ethanol. 0.004% of DPPH was prepared in ethyl alcohol and 3 ml of this solution was mixed with 3 ml of sample solutions. These solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm using UV Visible spectrophotometer. Alcohol (3 ml) with DPPH solution (0.004%, 3 ml) was used as blank.

The optical density was recorded and % inhibition was calculated using the formula given below:

$$\text{Percentage (\%) Inhibition of DPPH (\% AA)} = A - B \times 100$$

Where A= Optical density of the blank and B= Optical density of the sample.

The stock solution 1 mg/ml of ethanol was prepared. The required dilutions 0.1 mg/ml to 0.9 mg/ml were prepared by appropriate dilutions. The optical density and percent antioxidant activity were calculated.

Optical Density and Percent Antioxidant Activity for methanolic extract of *X. Strumarium* Leaves :(O.D. of Blank DPPH = 0.595)

Table 2: Antioxidant activities for ethanolic extract of *X. Strumarium* Leaves

S. N	Conc.mg/ml	O.D. of *X. strumarium*	%AA *X.Strumarium*
1.	0.01	0.388	34.78

2.	0.02	0.342	42.52
3.	0.03	0.329	44.70
4.	0.04	0.309	48.06
5.	0.05	0.278	53.27
6.	0.06	0.265	55.46
7.	0.07	0.240	59.66
8.	0.08	0.202	66.05
9.	0.09	0.172	71.09
10.	0.1	0.143	75.96

IC_{50} = 0.061mg/ml.

Calculation of IC_{50} Value for *X. strumarium leaves* extract = max - ½ (max-min)
=75.96 - ½ (75.96 – 34.78)
= 75.96 – 20.59
= 55.37

IC_{50} value from graph corresponding to *X. strumarium* leaves extract is 0.061 mg/ml

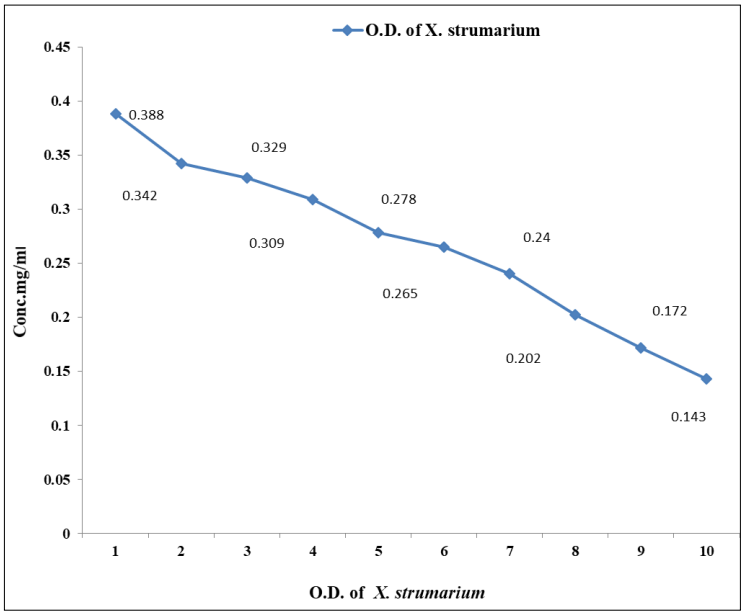

Fig 3: Decrease in Optical Density of Sample with Increase in Concentration of ethanolic Extract of *X. Strumarium* Leaves

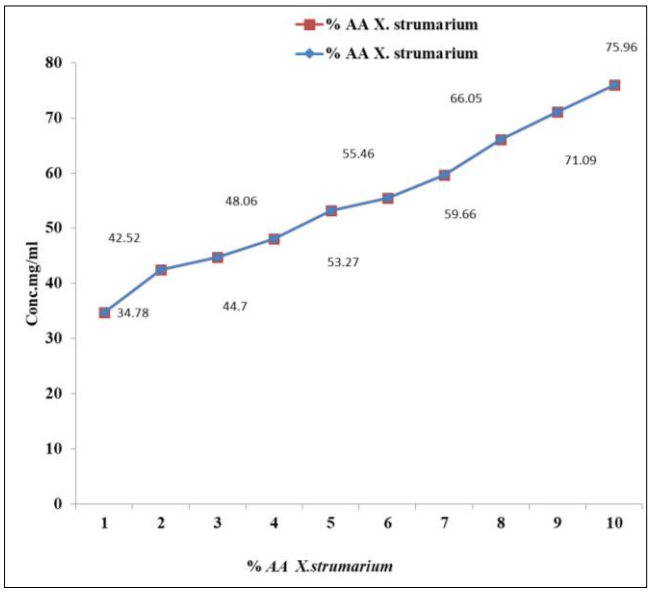

Fig 4: Increase in Percent Antioxidant Activity with Increase in Concentration of ethanolic Extract of *X. Strumarium* Leaves

Conclusion

In conclusion, it can be interpreted that topical application of *Xanthium strumarium* has a positive influence on different phases of wound healing, including wound contraction, fibroblastic deposition, angiogenesis and therefore, has a beneficial role in wound healing. However, identification and elucidation of the active constituents in this plant may provide useful leads to the development of new and effective drugs against different types of wounds. The plants are considered as very important natural source according to phytopharmacologists for discovering new pharmaceutical products having therapeutic benefits with no side effects. Numbers of Herbal drugs are known to treat various ailments. In present investigation, objective of the study was to extract the *Xanthium strumarium* L. leaves and evaluate its phytochemical and wound healing potential along with antioxidant property.

After that the extracts were subjected to in vitro antioxidant activity by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay activity. The methanol extract showed significant DPPH activity with the IC_{50} value of $55.37 \pm 0.61 \mu g\ mL^{-1}$.The ethanolic extract of *Xanthium strumarium* leaves had shown activity from slight to mark which was dose dependent high dose of extract (400mg) showed the marked increases in blood vessels. Angiogenesis plays a crucial role in wound healing by forming new blood vessels from preexisting vessels by invading the wound clot & organizing into a microvascular network through the granulation of tissue.

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