Research Article

# Evaluation of Wound Healing Potency of *Cassia auriculata* Flower Extracts Using Chick Embryo Wound Model

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**ABSTRACT**

*Cassia auriculata* Linn. is a plant reported for use in the Ayurvedic and Siddha system of medicine for treating various diseases. The study presented was an attempt to screen the ethanol and aqueous extracts of the flowers for wound healing potency using chick embryo wound model developed as part of the study. As evident from the experimental data the ethanol extract showed good dose-dependent healing potency. The ethanol extract at 500 µg concentration showed increased wound contraction by 50% compared to the negative control model in the chick embryo chorioallantoic membrane excision wound model. The results were statistically significant when compared to the control groups ($p<0.05$). The extract was also found to have better angiogenic properties from neovascularization studies. The chick embryo wound model has been reported to be a reliable model and could be used as an alternative to animal models for preliminary screening of compounds with wound healing potency.

**Keywords:** Angiogenesis, *Cassia auriculata*, Chick embryo, Chorioallantoic membrane, Wound healing.

## INTRODUCTION

Medicinal plants are important not only as therapeutic agents but also as health maintaining agents. Reports from World Health Organization (WHO) suggest more than 80% of the world's population relies on traditional medicine for their primary health care. Herbal medicines have long histories of use, ease of administration, low cost and excellent safety records. *Cassia* is a genus of indigenous medicinal plants of which the species *Cassia auriculata* has large biodiversity in south India. This plant is known to contain various active principles of therapeutic values and possesses biological activities against a number of diseases.[1,2]

*C. auriculata* commonly known as "avaram", is a shrub belonging to the Caesalpiniaceae family. The individual parts of the plant can be used for the treatment of various disorders in humans. Among the different parts, the plant is famous for its attractive yellow flowers which are found to contribute to the various biological activities of the plant. The plant has been reported to possess several biological properties like hepatoprotective, anticancer, antioxidant, antidiabetic, anti inflammatory and antimicrobial properties.[3-9]

Several phytochemicals like alkaloids, tannins and Saponins in ethanol extracts of certain plants have been reported to be effective against pathogens like *Candida albicans* which is a noted fungal pathogen in burn wounds. In addition phenolics, alkaloids and terpenoids exhibit excellent anti inflammatory activity, through any of the several cellular mechanisms like, altering the activities of immune cells involved in inflammatory reaction like the macrophages and neutrophil, modulating the proinflammatory gene expression, or by regulating the functional expression of the inflammatory enzymes.[10-12]

The reports on wound management conclude on abnormal microbial load, necrosis, trauma and edema and the vascular insufficiency resulting from them as the major factors behind complications and delay in wound healing. The phytochemicals with the above said properties can thus reduce the local slack up factors like repeated long time inflammatory necrosis and edema in the wound and the infection, accelerating the healing process. The wound healing potency of the flower extracts were studied in this paper owing to scanty reports in this area.[13]

Wound healing in mammalian tissues is a natural process of restoring the cellular structures and tissue layers, comprising three distinct phases: the inflammatory phase, the proliferative phase, and the remodeling phase; the main events being chemotaxis, phagocytosis, neocollagenesis, collagen deposition, angiogenesis and re-epithelialization. These phases and their bio-physiological functions must occur at the right time in proper sequence and continue for a specific duration at an optimal intensity. However, angiogenesis and re-epithelialization play a major role in the successful completion of the process. In spite of the fact that many mammalian animal models have been widely used in wound healing studies, several limitations for routine use of such models render them non-viable. Most important being, ethical constraints, cost and time consumption for preliminary screening and quantitative assessments of the crude extracts and phytoconstituents. Also, the molecular mechanism behind wound healing process is

difficult to explore due to the lack of proper models. These issues can be overcome by using models like chick embryo, zebra fish, drosophila, among others which serve as alternatives to the animal models. In the present study chick embryo has been used as a model to screen the wound healing potential of *C. auriculata* flower extract[14,15].

## MATERIAL AND METHODS

### Plant material

Flowers of *C. auriculata* were collected from Poondi, Tiruvallur district, Tamil Nadu, India in the month of October 2012. The plant material was identified and authenticated by taxonomist, Dr. P. Jayaraman at Plant Anatomy Research Center - herbal PARC, Chennai. The dust free flowers were shade dried in open air and ground to a coarse powder.

### Preparation of plant extracts

Fifty grams of the powder was extracted with ethanol and was filtered through eight layers of muslin cloth. The procedure was repeated twice with 250 ml of ethanol each. The pooled extracts were concentrated by evaporation. The residue was stored in stock vials for further use. Aqueous extracts were prepared by heating (45-55°C) 50 g of the powder with 300 ml of distilled water and repeating it twice with fresh water each time. The extracts were pooled, concentrated by evaporation and stored as before. The prepared extracts were qualitatively assessed for phytochemicals by standard methods (Table 1).[8]

### Embryo collection

Fertilized white shell eggs were purchased from Tamilnadu Veterinary and Animal Science University, Madhavaram Milk Colony, Chennai. The outer surface of the embryos were cleaned with 75% ethanol and incubated at 37°C throughout the study.

### Preparation of saturated filter disk for wound assay

Whatman No. 1 filter paper was purchased from Millipore. Small disks were generated using a standard 5 mm hole puncher, sterilized by autoclaving and stored for further use. The pre-sterilized filter disks were saturated with different concentrations of the crude extract, from 100-500 µg/ml, and the control solutions. Diclofenac sodium (50 µg/ml) in 4% ethanol and sterile saline were used as positive and negative controls respectively.[16]

### Wound assay

All dissection tools used in the assay were sterilized using 75% ethanol before use. The embryos were incubated for 11 days to allow good maturation of the chorioallantoic membrane. On day 12 of incubation the outer shell was wiped with 75% ethanol to sterilize the surface. Under aseptic conditions a tiny hole was made carefully in the egg shell with a needle and a small window of the shell was cracked open exposing the opaque inner shell membrane (Figure 1). About 0.5-1 ml sterile saline was added to the inner shell membrane to make it translucent. This layer was then peeled to visualize the CAM layer. The CAM layer was pulled gently by using sterile forceps and an excision wound of approximately 3 mm diameter was created in the CAM layer by using a small dissecting scissor. The drug saturated discs were then placed on the CAM of the embryos labeled with the corresponding concentrations and controls. The window on the egg shell was covered with para film and the eggs were returned to the incubator. Measurements of wound closure were made on alternative days up to day 5 of observation post wounding.

The wound closure was measured as wound contraction percentage (WC %) by using the formula.[17]

$$WC\ \% = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

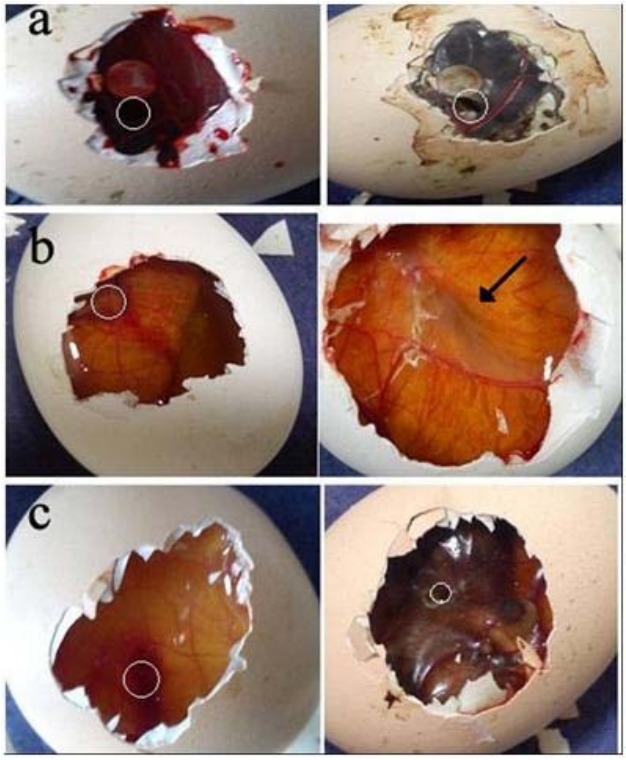


**Figure 1:** Treatment of the established wound model (a) negative control (saline), (b) positive control (Diclofenac sodium-50 µg/ml) and (c) treatment with the ethanol extract (500 µg/ml).

### Angiogenesis assay

The vascular network forms well in the developing embryo by 7 days of incubation. On day 8 of incubation the outer shell was wiped with 75% ethanol to sterilize the surface. Under aseptic conditions a tiny hole was made carefully in the egg shell with a needle and a small window of the shell was cracked open. The CAM layer was exposed as above. The drug saturated disks were then placed carefully on the CAM vasculature of the embryos labeled with the corresponding concentrations and controls and the egg shell window closed with

parafilm before incubation. Morphometric evaluation of the blood vessels on the CAM was done on alternative days of observation in terms of number of new vessels and thickness. The results were photographed on each observation and recorded (Figure 2).

### Statistical analysis

The collected data were analyzed with SPSS 16.0 version. The values are presented as mean with Standard Deviation. To find the significance with positive controls, One-Sample Wilcoxon Signed Rank Test was used. In the above statistical tool the probability value $p<0.05$ was considered as significant level.

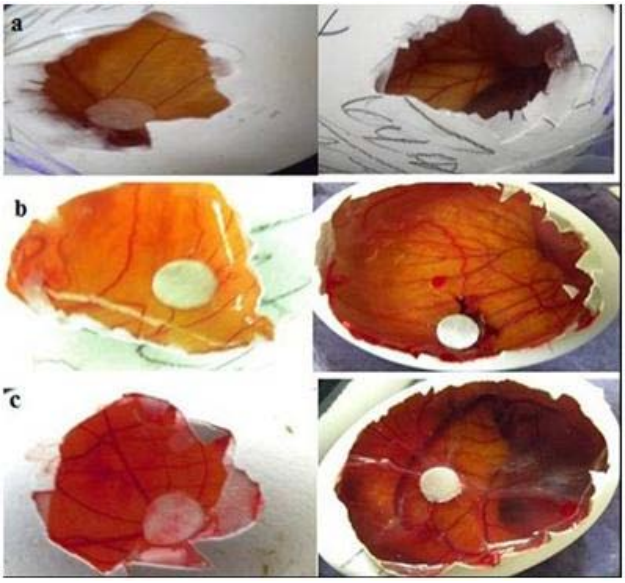


**Figure 2:** Neovascularization observed on the CAM layer of the treated models. (a) positive control (Diclofenac sodium-50 µg/ml), (b) negative control (saline), and (c) ethanol extract treatment (500 µg/ml)

### Gas chromatography-mass spectrometry analysis of plant extracts (GC-MS)

The ethanol extract with better wound healing activity was taken for further analysis to get the chemical definition. The analysis was performed using a Shimadzu GC/MS (GC-17A) equipped with a ZB-1 MS fused silica capillary column (30 m × 0.25 mm ID, film thickness 0.25 µm).

## RESULTS AND DISCUSSION

The results of the wound assay showed that % wound closure was dose-dependent with the maximum being at 500 µg/mL concentration. At 500 µg/mL concentration the ethanol and aqueous extracts showed 50% and 26.6% wound contraction compared to the positive control which showed 83.33% and the negative control which showed no significant wound closure (Table 2).

Angiogenesis was morphometrically analyzed and tabulated by counting the number of blood vessels in various treatments. Both the extracts promoted an increase in number of blood vessels compared to saline controls. The ethanol extract was more angiogenic in terms of increase in number and thickness of blood vessels than the aqueous extract (Figures 3 and 4).

**Table 1:** Preliminary phytochemical screening of the flower extract of *C. auriculata*

| Tests | Ethanol extract | Aqueous extract |
|---|---|---|
| Carbohydrates | + | + |
| Reducing sugars | + | + |
| Saponins | + | - |
| Flavonoids | + | + |
| Alkaloids | + | - |
| Tannins | + | + |
| Phenolic compounds | + | + |
| Steroids | + | + |

'+' indicates presence; '-' indicates absence

**Table 2:** Measurement of internal diameter (ID in mm) and wound contraction percentage (WC %) on day 5 post treatment with different concentrations of the two floral extracts of *C. auriculata*.

| Treatment | ID (mm) | WC % |
|---|---|---|
| Positive control (Diclofenac sodium 50 µg/ml) | 0.5±0.02* | 83.33 |
| Negative control (saline) | 3±0.01* | 0 |
| **Ethanol extract** | | |
| 100 µg/ml | 2.8±0.09* | 6.667 |
| 200 µg/ml | 2.7±0.04* | 10 |
| 300 µg/ml | 2.3±0.28* | 13.33 |
| 400 µg/ml | 1.9±0.10* | 33.33 |
| 500 µg/ml | 1.3±0.14* | 50 |
| **Aqueous extract** | | |
| 100 µg/ml | 2.6±0.11* | 13.33 |
| 200 µg/ml | 2.4±0.08* | 16.67 |
| 300 µg/ml | 2.4±0.16* | 16.67 |
| 400 µg/ml | 2.2±0.10* | 23.33 |
| 500 µg/ml | 2.0±0.10* | 26.67 |

ID Values presented are mean with standard deviation where n=6; $p<0.05$ was considered statistically significant compared to the control group.

The GC-MS analysis of the ethanol extract revealed twenty four compounds. The retention time and percentage peak of the individual compounds are presented (Table 3).[18]

The results indicate that the wound healing potency of the extracts significantly increases with increasing concentrations, both in terms of wound contraction and angiogenesis, which was comparable with the positive control Diclofenac sodium (50 µg/ml). The ethanol extract showed good healing results compared to the aqueous extract which can be attributed to its phytoconstituents.

The preliminary phytochemical analysis of the ethanol extract revealed the presence of phenolic compounds, tannins, Saponins, alkaloids, steroids and flavonoids whereas the aqueous extract showed the presence of phenolic compounds, flavonoids and tannins. Flavonoids have proved anti inflammatory activities. Inflammation being the first phase of wound repair mechanism has to be controlled within a day or two when the actual healing process begins. The extract confers this anti inflammatory trait to the wounded tissue which accelerates the healing process. Other compounds like terpenoids, tannins, Saponins, cardiac glycoside, steroids have proved antimicrobial activity that eliminates the heavy microbial load at the wound site, avoiding prolonged inflammation. The phenolic and tannin compounds have wound healing property in that tannin extracts have been reported to up-regulate the expression of vascular endothelial growth factor which enhances angiogenesis and wound contraction. [9,19,20]

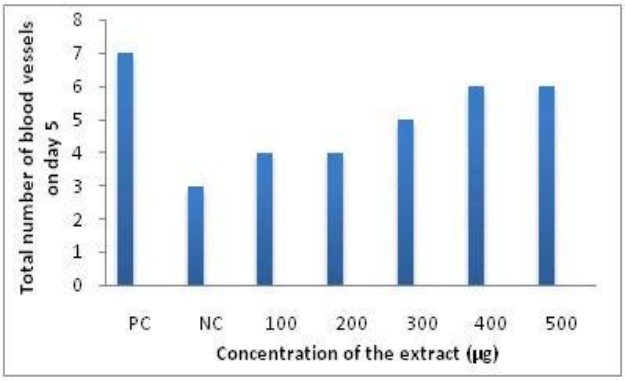


**Figure 3:** Increase in number of blood vessels was noted in the chick embryo models treated with different concentration of ethanol extract of *C. auriculata* compared to the positive (Diclofenac sodium 50 µg) and negative (saline) controls on day 5 post treatment.

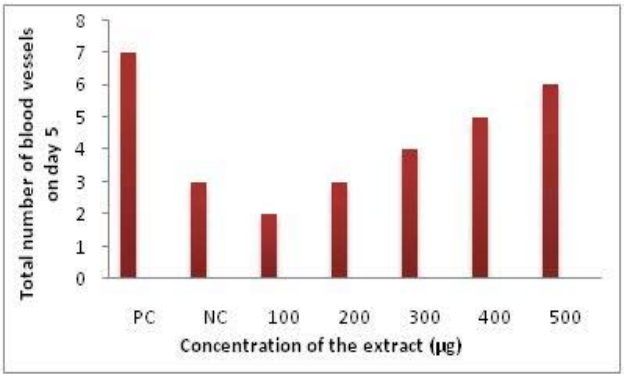


**Figure 4:** Increase in number of blood vessels was noted in the chick embryo models treated with different concentration of aqueous extract of *C. auriculata* compared to the positive (Diclofenac sodium 50 µg) and negative (saline) controls on day 5 post treatment.

Further GC-MS analysis of the ethanol fraction (Fig. 5) revealed 24 compounds, the major being benzofuran and 2,3-dihydrobenzene (peak 2), resorcinol (peak 3) and n-hexadecanoic acid (peak 4 ). Resorcinol has a wide range of wound healing applications. It has recorded use as an antiseptic and disinfectant, in ointments treating chronic skin diseases such as psoriasis, hidradenitis suppurativa and eczema. It has been the active ingredient used in over-the-counter topical acne treatments at about 2% and at higher concentrations in prescribed treatments like for gastric ulcers. Resorcinol polymerized gelatin-formaldehyde bioadhesive glue has been reported to accelerate the healing process in suture wounds. [21]

**Table 3:** GC-MS profile of the ethanol extract

| Phytochemical compound | Retention time (min) | Peak area % |
|---|---|---|
| Cyclohexanol | 8.577 | 0.58 |
| Levomenthol | 8.577 | 0.58 |
| Benzofuran | 9.143 | 4.29 |
| Resorcinol | 10.043 | 4.29 |
| 1,3:2,5-Dimethylene-l-rhamnitol | 12.178 | 0.78 |
| Hexadecanoic acid | 15.257 | 58.36 |
| Tridecanoic acid | 18.133 | 0.78 |
| Hentriacontane | 19.512 | 0.48 |
| Octacosane | 19.512 | 0.48 |
| Octadecadienoic acid | 19.512 | 0.48 |
| n-Hexadecanoic acid | 19.496 | 0.75 |
| Hexacosane | 19.512 | 0.16 |
| Heptadecyne | 20.064 | 0.33 |
| Eicosane | 21.122 | 0.19 |
| Methylthieno(3,2-b)pyridine | 20.122 | 0.27 |
| Hentriacontane | 21.122 | 0.19 |
| Benzo(h)quinoline- 2,4-dimethyl | 22.940 | 0.18 |
| Cyclotrisiloxane, hexamethyl | 22.284 | 0.19 |
| 1H-Indole, 5-methyl-2-phenyl | 22.284 | 0.19 |
| Ethyl 8-methyl-1,3-dioxo-2-phenyl | 23.027 | 1.43 |
| Benzene, (ethelynoxy) | 23.027 | 1.43 |
| Bis(2-ethylhexyl)-phthalate | 23.172 | 1.43 |
| 4-Hydroxyphenylpyruvic acid | 23.173 | 0.59 |
| 3,4Dimethoxymand-elic acid | 23.898 | 0.82 |

n-Hexadecanoic acid is said to inhibit phospholipase A(2) which otherwise results in ester bond hydrolysis of membrane phospholipids inducing inflammation in tissues. This anti inflammatory property of the compound in medicated oils is exploited for treatment of rheumatic symptoms in Ayurveda. The antioxidant and antimicrobial

properties of benzofuran family of compounds have been well reported, a contribution to the elimination of wound microbial load that otherwise results in slow healing. [22]

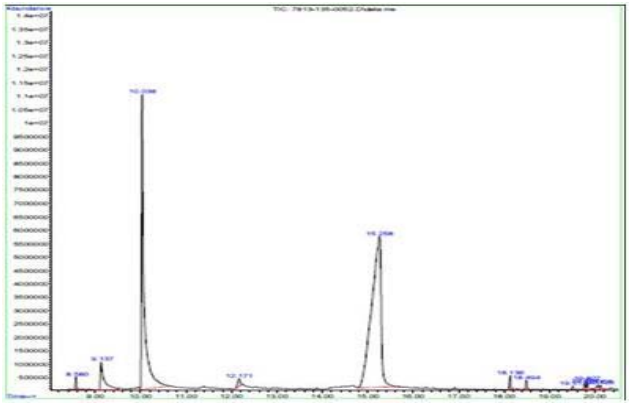

**Figure 5:** GC-MS profile of ethanol extract of *Cassia auriculata* flowers.

The present study reports a novel way of using chick embryo CAM model in preliminary screening of the phytocompounds wound healing potency. The results reveal the wound healing potential of *C. auriculata* flower extracts. This potential could be due to the ability of the phytoconstituents in the extract to regulate any of the proteins or chemotactic factors involved in the healing process at the molecular level as discussed above. The evaluation of the molecular mechanism behind this potency requires further studies in the established model which can be extended to wounds of different types and severity.

## CONCLUSION

The established chick embryo wound model could serve as an alternative to the *in-vivo* animal wound models for preliminary screening of wound healing phytocompounds. The experiment was designed based on the wound contraction and angiogenic traits expected to be possessed by compounds with wound healing potency. Of the two extracts tested ethanol extract of the *Cassia auriculata* flowers was found to possess excellent wound healing potency which was dose-dependent. The healing potency of the plant was evident from the phytoconstituents profile obtained from the GC-MS analysis. Further molecular mechanism behind the activity could be determined using the established model with the knowledge of the target protein or other factors involved in the complex healing pathway.

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