Discussion
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The aqueous extract of the freshly plucked *Tridax procumbens* leaves showed the extraordinary ability of healing wounds in rats. When the juice obtained from the leaves was applied to deep cuts or badly bleeding wounds it not only stopped the bleeding immediately but also reduced the inflammation. The wounds were healed without leaving any scar. Even though there is no record of the use of this extract for healing wounds in modern medicine, there have been numerous instances where the extract has been used for wound healing purposes in traditional system of medicine. A search of literature showed that others have also observed the wound healing ability of the leaf extracts, but the mechanism of action was largely not studied (154, 155). We therefore focused our attention first on identifying the components present in the aqueous extract of leaves, which help in wound healing process and then we have studied their mechanism of action. We have analyzed both aqueous as well as organic extracts of *Tridax procumbens* leaves so that comparison of their wound healing properties can be made.

Wound healing is a complex physiological process involving inflammatory response, migration of different types of cells to the wounded site, matrix synthesis, and deposition of collagen and re-epithelization of the wounded tissue (156). Neutrophils are the first leukocytes to arrive at the wounded site for controlling local infection of bacteria through endocytosis as well as the release of lysosomal enzymes (157). Fibroblasts and macrophages then infiltrate the wound to initiate reconstruction. Fibroblasts also produce collagen that participates in the healing process (158).

Several cytokines like the platelets derived growth factor (PDGF), transforming growth factor beta (TGF-β1) and fibroblast growth factor (FGF) play important role in the wound healing process. Besides the cytokines, cell adhesion molecules are also known to regulate cell functions such as proliferation, migration and matrix synthesis. TNF-α, IL-1, IL-4, IL-8, and TGF-β1 have been detected in the wounded site during wound healing process. The cell adhesion molecules such as vascular cell adhesion molecules (VCAM), E selectin, endothelial leukocyte adhesion molecules (ELAM) and intercellular adhesion molecules (ICAM) are reported to be expressed on
different type of the cells to a very high level with the onset of healing process. TNF-α induces the activation of endothelial cell to express ICAM and VCAM (159).

Therefore it would be interesting to assess whether the aqueous extract of *Tridax procumbens* leaves promote any of the cellular processes and induce secretion of some type of the cytokines known to be involved in wound healing. While testing the wound healing property of the aqueous and organic extracts and their different fractions on actual wounds on rats, we have also tested them for their ability to induce secretion of IFN-γ by human PBMC which is a measure of activation of innate immune system, secretion of IL-8 which being a chemokine measures the ability of the extracts to promote cellular migration and secretion of TNF-α which increases synthesis of cell adhesion molecules known to be involved in wound healing process and TGFβ1 which helps in various repair process.

The aqueous extract of *Tridax procumbens* leaves was prepared at room temperature using normal saline. Protein estimation of aqueous extract showed the presence of very less quantity of protein (160). Therefore the proteins were concentrated by acetone precipitation and the precipitated proteins were dissolved in small quantity of PBS (pH 7.2). Analysis of protein profile of the aqueous extract by using 15% SDS-PAGE showed that very few proteins were present in the saline extract of the leaves. Three prominent bands, one at about 80 kDa and two others at 22-25 kDa besides many minor bands, between 120 kDa to 10 kDa could be seen (fig.-2 lane 1). In a bid to find out whether some of these proteins are involved in wound healing process or not, the aqueous extract was subjected to trypsinization and then tested for their ability to help in wound healing. To find out whether trypsinization was effective or not the aqueous extract after trypsinization was analyzed by 15% SDS-PAGE (fig.-2 lane 2). It was observed that the proteins were degraded to small fragments only when large excess of trypsin was used and the sample was incubated for 24 hours at 37°C. But the trypsinized aqueous extracts retained their wound healing property as measured by healing of wounds on rats as well as induction of secretion of IFN-γ, TNF-α and IL-8 by human PBMC.
To test the thermal stability of the molecules involved in wound healing the extract was boiled for 15 minutes at 98°C. It destroyed its wound healing properties. On dialysis, using the membranes of 12kDa cut off, the wound healing properties of the extract was lost. Therefore, it appeared that the molecules responsible for wound healing property of the aqueous extract are not protein and are heat labile and are of low molecular weight. Therefore fractionation of molecules present in the aqueous extract before and after trypsinization was carried out by using sephadex G-100 chromatography (fig.-3). The fraction No.-3 obtained from the crude extract as well as the trypsinized extract showed wound healing property as measured by healing of rat wounds and induction of secretion of IFN-γ, TNF-α and IL-8. This supported our earlier observation that the molecules responsible for helping the wound healing process are trypsin resistant and small molecular weight entities. The trypsinized crude extract was subjected to Con.A sepharose fractionation to see if they are glycosylated molecules or not. It was seen that the wound healing activity resides in molecules, which bind to Con.A sepharose (fig.-4).

In order to establish whether the molecules responsible for wound healing can be extracted from the leaves with organic solvents or not, different solvents such as hexane, ethyl acetate, methanol and acetonitrile at room temperature as well as at their boiling points were used. For separating and visualizing the extracted molecules thin layer chromatographic analysis using different combination of various solvents were used. It was observed that ethyl acetate both at room temperature (25°C) as well as it’s boiling point (77°C) using Soxhlet apparatus, extracted maximum number of molecules from the dried leaves powder (fig.5 and 6). The best solvent system for separating these molecules on TLC was found to be the mixture of hexane:acetone; 60:40 (v:v). The ethyl acetate crude extract of the dried leaves were subjected to silica gel column chromatographic separation and the crude extract as well as the fractions (1, 2&3) obtained after silica gel chromatography were analyzed by TLC and HPLC (fig.7, 8&9).

It is known that bacterial invasion of wounded site delays the healing process and their control helps in the healing of the wound. Any compound, which has anti-bacterial property, can contribute to the
healing process. Therefore, we tested the antibacterial activity of the crude aqueous extract as well as the G-100 fractions and Con. A bound fraction of the trypsinized crude aqueous extract using eight different bacterial strains (fig. 10A, B, C and D). None of the fractions tested showed any significant antibacterial activity. Under similar conditions the organic solvent extract also did not show any activity (fig.11 A, B, C and D). Since wound healing requires cellular proliferation any compound which inhibits this process will interfere with the healing of wound. Therefore the anti-proliferative activity of crude aqueous extract and its different fractions as well as the organic solvent extract and its different silica gel fractions were tested using A549 and KB tumor cell lines (fig.12A&B). None of the aqueous as well as organic solvent fractions showed any significant anti-proliferative activity.

Wound healing is a complex cellular process, which involves migration of cells and repairing of the wounded site. In this cytokines and various growth factors take part. Therefore the ability of extracts of *Tridax procumbens* leaves and their fractions to induce secretion of different cytokines like IL-2, IFN-γ, TNF-α, TGFβ1 and chemokine like IL-8 by human PBMC were tested. For determination of optimal dose of the extracts and their fractions needed for induction of different cytokines, PBMC isolated from the blood of 5 healthy individuals were cultured individually in the presence of different concentrations of crude aqueous extract of *Tridax procumbens* leaves, its sephadex G-100 fraction-3, Con. A sepharose bound fraction and crude ethyl acetate fraction.

It was observed that the crude aqueous extract at 15μg/ml, the G-100 fraction at 10μg/ml and the Con. A sepharose bound fraction at 4μg/ml induced maximum level of IL-2 secretion by human PBMC (fig.-13). This indicate that the aqueous extract and its fractions has immunomodulatory activity on human PBMC. But the crude ethyl acetate extract did not show any such effect over a wide range of concentrations (2-25μg/ml). Therefore it become necessary to find out the optimal dose of the aqueous extract and its fractions needed for the secretion of other cytokines. When the induction of secretion of IFN-γ by human PBMC in presence of different concentrations of crude aqueous *Tridax procumbens* leaves extracts and their fractions
were studied, it gave very similar type of dose response (fig.-14). IL-4 is an antagonistic cytokine of IFN-γ. Its presence polarizes the immune response to Th2 type. Therefore when the effect of the crude extracts and its fractions were tested using human PBMC we found that the crude aqueous extract at 15μg/ml, G-100 fraction-3 at 15μg/ml and Con.A sepharose bound fraction at 10μg/ml induced optimal secretion of IL-4 by them (20-45pg/ml). But the crude ethyl acetate extract at different concentrations ranging from 2μg to 25μg/ml did not do so (fig.-15). Similarly the optimal dose of crude extract as well as their different fractions needed to induce secretion of TNF-α, an important proinflammatory cytokine, TGFβ1, a growth factor and IL-8, a chemokine by the human PBMC was determined (fig.16-18).

TNF-α and TGFβ1 are known to promote wound healing by induction of synthesis of cell adhesion molecules, which help in attachment of different type of cells to each other. IL-8, as a chemokine, would help cells bearing IL-8 receptors to migrate towards the source of its production. Since the dose response analysis indicated that the aqueous extract and its different fractions induced synthesis and secretion of these cytokines by human PBMC, we became interested to study their effect using PBMC from a larger number of human volunteers. Since IL-2, besides many other functions, promotes proliferation of T cells we analyzed the ability of crude aqueous extract, G-100 fraction-3, Con.A sepharose bound fraction and the crude ethyl acetate fraction to induce its secretion by PBMC taken from 15 healthy human volunteers. The data presented in fig. 19 gives the value of IL-2 secreted by 2.5x10⁵ PBMC of different individuals in the presence of optimal dose of different extracts and fractions. The crude aqueous extract and its two fractions induced secretion of IL-2 to a much higher level (120-250pg/ml) than the medium control (20-30pg/ml). The crude ethyl acetate extract did not induce secretion of significant quantity of IL-2 (30-60pg/ml). This authenticated our earlier observation obtained during determination of dose response for these fractions.

IFN-γ plays an important role in activation of macrophages and phagocytosis and clearance of bacteria. Like the IL-2 the crude aqueous extract and its G-100 fraction-3 as well as Con.A sepharose bound fraction induced secretion of moderately high levels of IFN-γ (fig.20). This may be helping bacterial clearance by
macrophages. Similar results were obtained for IL-4 (fig.21), TNF-α (fig.22), TGFβ1 (fig.23) secretion by human PBMC. Therefore the crude aqueous extract as well as it’s low molecular weight G-100 fraction-3 and Con.A sepharose bound fraction by inducing secretion of these cytokines may be helping in wound healing process as observed by us using wounds in rats. Our earlier observation that the activity resides in non-proteinaceous small molecular weight molecules is also authenticated by observation that such molecules are capable of inducing secretion of significant levels of these cytokines. Chemokine like IL-8 will attract cells bearing IL-8 receptors towards itself. Neutrophils are known to migrate to the wounded site in large numbers. This migration may be promoted by IL-8 secretion at the site. We found that the Con.A sepharose bound fraction of the aqueous extract induces secretion of significant quantity of IL-8 by the human PBMC (fig.24). Thus it would help in wound healing process by attracting a larger number of neutrophils which will clean up the wounded site.

From all these data it is clear that the aqueous extract as well as it’s low molecular weight sephadex G-100 fraction-3 and non-proteinaceous Con.A sepharose bound fraction are capable of modulating the immune system in a manner which would help in wound healing. Therefore we have tested their effect on neutrophils purified from human blood and measured the levels of TNF-α, IFN-γ and IL-8 secreted by them into the medium. It was observed that the crude aqueous extract and it’s G-100 fraction-3 as well as Con.A sepharose bound fraction induced secretion of TNF-α (600-1500pg/ml) (fig.25), IFN-γ (800-1600pg/ml) (fig.26) and IL-8 (500-1500pg/ml) (fig.27). Thus all these fraction are able to activate neutrophils and therefore may be able to help in the wound healing process. THP1 is a human monocytic cell line. Since macrophages are known to play a very important role in wound healing, any compound, which activates them, also may help in the wound healing process. Therefore we have tested the ability of the crude aqueous extract and two fractions as well as the crude ethyl acetate extract to activate THP-1 cell line to secrete TNF-α and IL-8. We found that the crude aqueous extract and it’s two fractions induced secretion of significant amount of TNF-α (fig.28) and IL-8 (fig. 29) in THP-1 cells. This is interesting and supports our earlier observations using human PBMC.
Since on activation human neutrophils express CD28 molecules on their surface, the ability of crude aqueous extract to induce expression of CD28 on neutrophils was tested by FACS using anti CD28 antibodies. It appears that CD28 expression is enhanced in human neutrophils after culturing with crude aqueous extract for 48 hours (fig.30). The same result was obtained when purified human neutrophils were cultured in the presence of Con.A sepherose bound fraction of crude aqueous extract of *Tridax procumbens* leaves (fig.31).

When expression of CD28 and IL-8 receptors on the surface of purified human peripheral blood neutrophils after culture with PMA, crude aqueous extract of the *Tridax procumbens* leaves and it’s G-100 fraction 3 and Con.A sepherose bound fraction were monitored by immunoprecipitation using anti CD28 (fig.32) and IL-8 receptor antibodies respectively, it was observed that CD28 and IL-8 receptors are expressed on the surface of neutrophils (fig.33). This clearly shows that the crude aqueous extract as well as it’s low molecular weight nonproteinaceous fraction can activate neutrophils (CD28 expression) and induce their migration to the wounded site (IL-8 receptor expression) which would then help in wound healing process.