CHAPTER 6
DISCUSSION
6.1 Method optimization

Method for anti-thrombotic and thrombolytic activity was standardized. For the method standardization, streptokinase was used as standard. Various concentrations of streptokinase were used in varying amount of blood and blood clots. The dose of streptokinase was optimized as shown in table no. 4.1 to 4.8. Streptokinase was dissolving clots and inhibiting clot formation effectively.

The concentration of streptokinase was 5000 units/ml. and volume of streptokinase varying from 0.1ml to 1.0 ml for both anti-thrombotic and thrombolytic activity.

The minimum concentration showing antithrombotic activity was 0.2ml (5kU/ml) and minimum concentration showing thrombolytic activity was 0.5ml (5kU/ml), hence these concentrations were taken as standard (i.e. 0.2ml for antithrombotic activity and 0.5ml for thrombolytic activity).

Streptokinase injection was chosen as standard for antithrombotic and thrombolytic activity. 5000 unit/ml strength was used as working concentration. The normal saline was used as diluents and vehicle for streptokinase and also used as blank.

The extraction of plants were done by maceration and concentrated over water bath and stored in airtight containers.

Normal saline was used as vehicle for these plant extracts also. Activity was done as method optimized.

Two dilutions of extracts were used for antiplatelet and thrombolytic activity i.e. 5x and 10x. These dilutions were made in normal saline.
immediately before the experiment. Varying amount of diluted extracts (0.1ml to 0.5 ml) was used for test of activity.

The antiplatelet activity was taken of all plant extracts by spectrophotometric method. Collagen was taken as platelet aggregation inducer and aspirin was used as standard for antiplatelet activity.

The principle of the method is when an agonist (i.e. collagen) was added in PRP, the platelets aggregate and settle down hence absorbs less light so the transmission increases and this is detected by the photocell.

The working concentration of collagen was taken 0.2mg/dl and .02ml of collagen was used for each tube as this was optimized amount and referred from published work. [1]

The concentration of aspirin and method of testing were optimized by given method (page 43) and dose was finalized was 0.4 ml of 0.9mg/dl (as the working concentration of aspirin reported is 100 µ M). [1]

The antiplatelet activity was taken by the method optimized and discussed above (page 46). The extracts were diluted 50x and 100x in normal saline and normal saline was also used as blank.
6.2 Antithrombotic activity

6.2.1 *Terminalia belerica*

The aqueous and alcoholic extracts of *Terminalia belerica* were inhibiting clot formation and their potency were dose dependent. The minimum concentration showing antithrombotic activity was 0.1mg/dl. 0.6mg/dl concentration of aqueous extract and 1.0mg/dl of alcoholic extracts were showing antithrombotic activity equivalent to 1KU/ml of SK. (Table 5.45)

6.2.2 *Nigella sativa*

*Nigella sativa* extracts (aqueous and alcoholic) were delaying the clot formation time. The minimum concentration showing antithrombotic activity was 0.1mg/dl. The maximum used concentration (1.0mg/dl) was not as much potent as SK for antithrombotic activity. It showed 30% active against 1KU/ml of SK. (Table 5.46)

6.2.3 *Nepeta hindostana*

Extracts of *Nepeta hindostana* were inhibiting clot formation effectively in all used concentrations. The minimum concentration showing antithrombotic activity was 0.1mg/dl. 0.4mg/dl concentration of aqueous and alcoholic extracts was shown antithrombotic activity equivalent to 1KU/ml of SK. (Table 5.47)
6.3 Thrombolytic activity

6.3.1 Terminalia belerica
All used concentrations of *Terminalia belerica* aqueous and alcoholic extracts were dissolving the clots before blank (maximum time monitored was 90 mins) but alcoholic extract was less potent than aqueous. The minimum concentration showing thrombolytic activity was 0.1mg/dl. The maximum used concentration (1.0mg/dl) was not as much potent as SK for thrombolytic activity. It was found 78% active against 2.5KU/ml of SK. (Table 5.45)

6.3.2 Nigella sativa
Aqueous extract of *Nigella sativa* was showing clot dissolution only at higher concentration. The alcoholic extract was not showing any remarkable clot dissolution in given time.
The minimum concentration showing thrombolytic activity was 0.5mg/dl. The maximum used concentration (1.0mg/dl) was found 54% active against 2.5KU/ml of SK. (Table 5.46)

6.3.3 Nepeta hindostana
*Nepeta hindostana* aqueous and alcoholic extracts were successfully dissolving clots under given test conditions but again alcoholic extract proved less potent than aqueous. The minimum concentration showing thrombolytic activity was 0.3mg/dl.
The maximum used concentration (1.0mg/dl) was found more potent than SK for thrombolytic activity. If we consider SK activity as 100% then *Nepeta hindostana* showed 141% active against 2.5KU/ml of SK. (Table 5.47)
6.4 Antiplatelet activity

6.4.1 Terminalia belerica
All used dilutions of *Terminalia belerica* aqueous were showing good antiplatelet activity.
Alcoholic extract was also showing remarkable antiplatelet activity but less potent than aqueous. (Table 5.48)
The minimum concentration showing antiplatelet activity was 0.5µg/ml.
The maximum used concentration (5.0µg/ml) was proved more potent than the aspirin used. If we consider aspirin activity as 100% then *Terminalia belerica* showed 117% active against 18µg/ml of aspirin. (Table 5.48)

6.4.2 Nigella sativa
*Nigella sativa* showed nominal activity, only maximum used concentration (5.0µg/ml) was showing bit remarkable activity in case of aqueous extract. The alcoholic extract doesn’t show any remarkable activity in all concentrations used. (Table 5.49)
The minimum concentration showing antiplatelet activity was 2.0µg/ml.
The maximum used concentration (5.0µg/ml) was found 67% active against 18µg/ml of aspirin.

6.4.3 Nepeta hindostana
*Nepeta hindostana* showed very good antiplatelet activity in both dilutions of aqueous and alcoholic extracts. The potency of response was greater than *Terminalia belerica*. Alcoholic extract proved less potent than aqueous.
The minimum concentration showing antiplatelet activity was 0.5µg/ml. The maximum used concentration (5.0µg/ml) was proved more potent than the aspirin used. If we consider aspirin activity as 100% then Nepeta hindostana showed 133% active against 18µg/ml of aspirin. (Table 5.50)

6.5 Ethanolic extract
Since all the above alcoholic extracts were taken by methanol and there was possibility to present some different compound in ethanolic extracts, hence ethanolic extracts were also taken by same method for all three plants and dilutions were made by same method.

Both thrombolytic and antithrombotic activities were tested by ethanolic extract by same methods.

The results for ethanolic extracts were same as methanolic extracts for all three plants. There was no any remarkable difference in activities of both extract. (Table 5.52, 5.53)
6.6 Fractionation

Both plant extracts showed expected results/activity. The rich (dark/thick) fraction from TLC was showing the activity. In case of *Terminalia belerica*, the fraction of higher Rf value was showing both antiplatelet and thrombolytic activity. (Table 5.56, 5.57, 5.60, 5.61)

In case of *Nepeta hindostana* aqueous extract, higher Rf value compound was showing distinguish activity. In alcoholic extract of *Nepeta hindostana*, the active fraction was present below few bands (i.e. medium Rf value). (Table 5.58, 5.59, 5.62, 5.63)

6.7 Characterization compound TB-01

**Compound TB-01** named Benzoyl – β –D – (4’→10’’ geranilanoxy) -xylopyranosides, was obtain as a brownish crystalline mass from chloroform: methanol (4:1) eluant. It’s responded positively to test for glycosides and showed IR absorption bands for hydroxyl groups (3401,3315 cm \(^{-1}\)), carbonyl function (1646 cm \(^{-1}\)) and unsaturation (1525 cm \(^{-1}\)). On the basis of mass and \(^{13}\)C NMR spectra, the molecular ion peak of **TB-01** was determined at \(m/z\) 394 corresponding to a xylopyranosides glycoside C\(_{22}\)H\(_{34}\)O\(_6\). The \(^1\)H NMR spectrum of **TB-01** showed three one-proton doublets at \(\delta\ 4.67\ (J=7.2\ Hz),\ 3.56\ (J=4.8\ Hz)\ and 3.52\ (J=5.7\ Hz)\) assigned to anomeric H-1’, H\(_2\)-5α and H\(_2\)-5β protons, respectively. It also showed the one proton multiplet from \(\delta\ 7.52\) to 6.98 and four two proton multiplet at \(\delta\ 2.56,\ 2.50,\ 2.11\ and 1.19\) assigned to H\(_2\)-5’’, H\(_2\)-4’’, H\(_2\)-6’’ and H-2’’ protons, respectively. The other sugar protons appeared between \(\delta\ 3.83\) -3.10. A two-proton doublet at \(\delta\ 3.31\ (J=6.1\ Hz)\) was ascribed to
methylene H_{2-10''} adjacent to the carbonyl function and two 2 proton doublet at δ 1.37 and 1.27. A three-proton triplet at δ 0.86 (J=6.5 Hz) was accounted to the methyl protons. The $^{13}$C NMR spectrum displayed signals for carbonyl carbon at δ 170.51 (C-6), anomeric carbon at δ 146.46 (C-1), other carbons between δ 90.31 – 62.65. On the basis of these result structure of TB-01 has been characterized as Benzoyl – β-D – (4’→10’’ geranilanoxy) – xylopyranosides. It is a new compound.

**IR ν_{max} (KBr):** 3401, 3315, 2916, 2864, 1646, 1525, 1435, 1384, 1215, 1156, 1033, 758 cm$^{-1}$.

**$^1$H NMR (MeOD):** δ 7.52 ( 1H, m, H-2), 7.46 (1H, m, H-6), 7.08 (1H, m, H-3), 7.05 (1H, m, H-5), 6.98 (1H, m, H-4), 4.67 (1H, d, J= 7.2 Hz, H-1’), 4.38 (1H, m, H-2’), 4.04 (1H, m, H-4’), 3.74 (1H, m, H-3’), 3.56 (1H, d, J= 4.8 Hz, H_{2-5α}), 3.52 ( 1H, d, J= 5.7 Hz, H_{2-5β}), 3.31 ( 2H, d, J= 6.1 Hz, H_{2- 10''}) 2.90 (1H, m, H-7’’), 2.58 (1H, m, H-3’’), 2.56 (2H, m, H_{2-5’’}), 2.50 (2H, m, H_{2-4’’}), 2.11 (2H, m, H_{2-6’’}), 1.37 (3H, d, J= 5.4 Hz, Me-9’’), 1.27 (3H, d, J=5.4 Hz, Me-8’’), 1.19 (2H, m, H-2’’), 0.86 (3H, t, J=6.5 Hz, Me-1’’).

**$^{13}$C NMR (MeOD):** δ 146.49 (C-1), 139.69 (C-2), 123.48 (C-3), 110.43 (C-4), 118.37 (C-5), 122.06 (C-6), 170.51 (C-7), 90.31 (C-1’’), 75.08 (C-2’’), 69.84 (C-3’’), 73.97 (C-4’’), 64.51 (C-5’’), 13.18 (C-1’’), 22.03 (C-2’’), 46.34 (C-3’’), 30.16 (C-4’’), 36.27 (C-5’’), 38.89 (C-6’’), 42.18 (C-7’’), 15.07 (C-8’’), 17.38 (C-9’’), 62.65 (C-10’’).

**ESI MS m/z (rel.int.):** 394 [M]$^+$ (C$_{22}$H$_{34}$O$_6$) (1.3), 237 (3.8), 141 (4.3).
6.7.1 Structure of isolated compound TB01:

Benzoic acid 5-(2-ethyl-6-methyl-heptyloxy)-3,4-dihydroxy-tetrahydro-pyran-2-yl ester