CHAPTER 6

Chapter-6 Discussion on results

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CHAPTER 6

Discussion on results

The Present investigation reported in this thesis was aimed to explore the folklore use of two medicinal plants *Shorea tumbuggaia Roxb.* and *Holostemma ada- Kodien Schult*. Ethnobotanical survey was carried out on medicinal plants listed by IUCN red list of threatened species found in southern Eastern Ghats of India. These medicinal plants were subjected for various phytochemical investigation and pharmacological evaluation and the results were reported in the previous chapter. This chapter discusses in detail on the results obtained on various investigations performed on crude extracts of STR and HAKS.

6.1. Preliminary phytochemical analysis

Various crude extracts extracted from STR and HAKS were subjected for preliminary phytochemical investigations as described in the earlier chapters. The result of the present investigation proves presence of steroid in the n-hexane extract of both the plants. The ethyl acetate extract of STR showed the presence of tannins, flavonoids and triterpenoids. Whereas ethyl acetate extract of HAKS showed the presence of tannins, flavonoids, anthocyanins and triterpenoids.

The methanolic extract of STR and HAKS showed the presence of carbohydrates, alkaloids, saponins, tannins, flavonoids,
triterpenoids and anthocyanins. Apart from the above mentioned phytoconstituents methanolic extract of HAKS was found to contain amino acids. Hydroalcoholic extracts of both the plants have showed presence of similar phytoconstituents. The phytoconstituents present are carbohydrates, flavonoids and triterpenoids.

6.1.1. Estimation of total phenolic content

Total phenolic content of the above mentioned extracts of STR and HAKS were performed using Folin-Ciocalteu method. The results showed that the methanolic extract has highest phenolic content when compared with other extracts. Among the two plants methanolic extract of HAKS was found to contain higher amount of phenolic content with 83.977 ± 5.132 GAE mg/g of dry material.

6.1.3. Estimation of total flavonoidal content

Total flavonoidal content of ethyl acetate, methanolic and hydro-alcoholic extracts of STR and HAKS were performed using aluminium nitrate-potassium acetate method. The results showed that the methanolic extracts have highest flavonoidal content when compared with other extracts. Among the two plants methanolic extract of HAKS was found to contain higher amount of flavonoidal content with 72.744 ± 1.528 QE mg/g of dry material.
6.2. *In-vitro* antioxidant studies

A large number of phytoconstituents have been reported for their antioxidant activity \(^{147}\). Many authors have reported the relation between antioxidant activity and polyphenolic compounds\(^{148}\).

The free radical scavenging effects of all the extracts on DPPH, nitric oxide, hydroxyl, superoxide anion, hydrogen peroxide radicals were carried out using the procedures discussed in earlier chapters. All the extracts were also screened for their metal chelating ability and ferric ion reducing power using standard procedures. The results of above mentioned investigations are discussed in this section.

6.2.1. DPPH radical scavenging activity

The present investigation proved that among all the extracts methanolic extract of STR and HAKS have significant antioxidant activity with an IC\(_{50}\) value of 46.80 and 63.64 respectively. Among the other extracts hydroalcoholic extract of STR and Ethyl acetate extract of HAKS have shown moderate antioxidant activity with an IC\(_{50}\) value of 84.45 and 116.724 respectively.

The results of the DPPH radical scavenging assay reveal that these extract are capable of scavenging free radicals in solution prevent initiation of free radical-mediated chain reactions by preventing the abstraction of hydrogen \(^{149-153}\). The results are shown in Table. 5.4 and 5.5 respectively.
6.2.2. Hydroxyl radical scavenging activity

Hydroxyl radical is an enormously responsive free radical formed in biological arrangements and has been concerned as highly destructive classes in free radical pathology, which are capable of damaging particle found in active cells\(^{154}\). Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity\(^{155}\).

The ability to reduce hydroxyl radicals appears to be unswervingly associated to the inhibition of dissemination process in lipid peroxidation and appears to be respectable scavenger of active oxygen species, thus reducing the rate of the chain reaction\(^{156}\). High molecular weight and the nearness of many aromatic rings and OH groups are most significant for the free radical-scavenging activity by phenolics than their specific functional groups\(^{157}\).

This reaction is sometimes called an iron-catalyzed Haber-Weiss reaction which can be written as:

\[
\text{Fe}^{2+} + \text{O}_2^+ \rightarrow \text{Fe}^{2+} + \text{O}_2 \quad \text{(metal reduction)}
\]

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{OH} + \text{OH}^- \quad \text{(Fenton reaction)}
\]

\[
\text{O}_2^+ + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH} + \text{OH}^- \quad \text{(Haber-Weiss reaction)}
\]

The present investigation proved that among all the extracts methanolic extract of STR and HAKS have significant antioxidant activity with an IC\(_{50}\) value of 79.250 and 56.477 respectively. Among the other extracts hydroalcoholic extract of STR and Ethyl acetate extract of HAKS have shown moderate antioxidant activity with an
IC$_{50}$ value of 97.088 and 96.076 respectively. The results are shown in Table 5.6 and 5.7 respectively.

6.2.3. Nitric oxide radical scavenging

Nitric oxide is a vital bio-regulatory molecule essential for numerous physiological progressions like immune response, control of blood pressure neural signal transmission, and control vasodilatation$^{118}$. Also nitric oxide plays an important role in many types of inflammatory processes in the body$^{158-160}$. In the present study leaves extracts STR and HAKS were studied for its inhibitory effect on nitric oxide production. Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by ME-STR, HAE-STR, EAE-HAKS and ME-HAKS were 26.507, 66.889, 98.495 and 40.661 when compared with standard reference ascorbic acid. The results are shown in Table. 5.8 and 5.9 respectively.

6.2.4. Superoxide anion radical scavenging

Superoxide radical is recognized to be very destructive to cellular components as a predecessor of the more reactive oxygen species (ROS), causative to tissue impairment and various ailments$^{161-164}$. The reaction mechanism is based on the generation of superoxide radical (O$_2^-$) by auto-oxidation of hydroxylamine hydrochloride in presence of NBT, which gets reduced to nitrite. The leaves extract of STR and HAKS shows the presence of potent superoxide radical scavenging activity. The highest activity was seen in ME-STR and ME-HAKS followed by EAE-HAKS and HAE-STR
extracts when compared with other extracts. They are also statistically significant when compared with standard reference compound BHA in terms of % inhibition and IC\textsubscript{50}. The results are shown in Table. 5.10 and 5.11 respectively.

6.2.5. Hydrogen Peroxide

The breakdown of hydrogen peroxide into water may arise by rendering to the following reaction

\[
\text{H}_2\text{O}_2 + \text{H}_2\text{O} + 2\text{e} \rightarrow 2 \text{H}_2\text{O}
\]

Since phenolic composites existing in the natural products are good electron donors, they may quicken the alteration of H\textsubscript{2}O\textsubscript{2} into H\textsubscript{2}O. However, H\textsubscript{2}O\textsubscript{2} in the existence of iron ions can create enormously sensitive OH\textsuperscript{165}.

The recognition of OH as such is extremely difficult, due to its very short life time. The capability of phenolic composite were known to produce effectively scavenge OH\textsuperscript{166}. Flavonoids (myrcetin, rhamnetin and quercetin) were also found to be OH scavengers. The efficiency of such compounds intensifications with increasing amount of hydroxyl groups attached to the aromatic B-ring\textsuperscript{167}.

The highest activity was seen in ME-STR and ME-HAKS (197.439 and 215.206) followed by EAE-HAKS and HAE-STR (292.022 and 280.161), percentage inhibition and IC\textsubscript{50} were statistically significant when compared with standard reference compound Gallic acid. The results are shown in Table. 5.12 and 5.13 respectively.
6.2.6. Metal chelating Ability

The chelating ability of Fe\(^{2+}\) by the crude extracts was estimated by ferozine quantitatively form complex with Fe\(^{2+}\) in existence of chelating agents, the complex formation is inhibited by reducing the intensity of the colour chromogen. The reduction allowed for measuring the amount of chelating activity of the coexisting chelator.

Thus, Fe\(^{2+}\) conversion metal ion have the capability to move single electron by asset of which it can allow the formation and propagation of many reactive radical\(^{121}\), the decrease in the intensity by the antioxidant present in the crude extracts may be due to the inhibition of ROS related with redox catalysis (metal) which interferes with chelating ability of the metal ions.

ME-STR and ME-HAKS (175.259 and 176.164) shows the presence of highest metal chelate activity. The potent antioxidant activity was observed in methanolic extracts of both the plant (STR and HAKS) when compared with other extracts and standard reference compound α-tocopherol in terms of percentage inhibition and IC\(_{50}\). The results are shown in Table 5.14 and 5.15 respectively.

6.2.7. Reducing power ability

Reducing power is accompanying with antioxidant property and assists as important replication on antioxidant activity \(^{168}\). Compounds with reducing power are specifying to electron donors and can diminish the oxidized intermediates of lipid peroxidation
progressions, so that can be used as primary and secondary antioxidants.

Presence of reducing materials causes the alteration of Fe $^{3+}$/ferricyanide complex to ferrous. Thus, formed pearl's Prussian blue at 700nm, it's possible to determine the concentration of Fe$^{3+}$. The reducing power of the extract was seen for the methanolic and hydro-alcoholic of STR and methanolic and ethyl acetate extract of HAKS were consider to have a potent activity when compared with reference standard ascorbic acid. The results are shown in Table. 5.16.

6.3. Pharmacological screening for the leaves extracts of STR and HAKS

6.3.1. Acute toxicity test

This investigation was performed as per the procedure discussed in the earlier chapters. The extracts were found to be safe upto 3500 mg/kg. b.w. Thus a safer dose of 100, 200 and 400 mg/kg. b.w was used throughout the investigation.

6.3.2. Anti-inflammatory activity

Anti-inflammatory studies were implemented by formalin persuaded paw oedema technique which is normally done for the valuation of anti-inflammatory action for natural products. It is well
recognized that the inhibition of formalin persuaded paw oedema in rats is one of the most appropriate test dealings for monitor of anti-arthritic and anti-inflammatory agent as it closely look like human arthritis by the injection of formalin subcutaneously into hind paw of rats produces restricted inflammation and pain\textsuperscript{169,170}.

Formalin influenced paw oedema in rats is one of the most suitable test procedures to screen the acute inflammation and it is supposed to be a biphasic occurrence.

Among the various phytoconstituents flavonoids have been reported in controlling inflammatory ailments and also the anti-inflammatory action is a communal property of numerous phenolic and terpenoids\textsuperscript{171}. Predominantly flavonoids are also reported for antioxidant, anti-inflammatory, anti-hepatotoxic, vascular protector, anti-tumor activity and anti-allergic\textsuperscript{172}. The anti-inflammatory properties of triterpenes were accredited to several mechanisms including inhibition of lipoxygenase and cyclooxygenase activities\textsuperscript{173}.

Introduction of formalin causes the variations in connective tissue metabolism which is a major biochemical occurrence throughout the course of inflammation. These differences are effects the relative alignment of various constituents of connective tissue such as hexosamine, hydroxyl proline, mucopolysaccharides, glycoprotein, and sialic acid\textsuperscript{174}. 

In the present examination, the formalin persuaded paw oedema was performed for the crude leaves extract of both plants methanolic (ME-STR) and hydro-alcoholic (HAE-STR) *Shorea tumbuggaia* Roxb. (STR) and methanolic (ME- HAKS) and ethyl acetate (EAE-HAKS) *Holostemma ada-Kodien Schult.* (HAKS) extract in the dose ranging from 100, 200 and 400 mg/kg b.w. The results were statistically evaluated by one way ANOVA, graphpad Prism 6.0 version Dunnett’s multiple comparisons test version.

The result was evident that the methanolic extract of both STR and HAKS showed highest amount of percentage inhibition after injection of toxicant formalin (0 to 3h). When compared with control and positive control standard diclofenac sodium (30 mg/kg b.w), the percentage inhibition of ME-STR and ME-HAKS at high dose (400mg/kg) was 69.291 ± 3.341% and 77.559 ± 2.4787% these values are considered to be significant with P value less than 0.0001 at 3h.

HAE-STR and EAE-HAKS at high dose (400mg/kg b.w) percentage inhibition 48.819 ± 3.556 and 52.755± 2.111 at 3h with P value of 0.001 and positive control showed percentage inhibition 52.756 ± 1.494, at 3h with P value less than 0.001 when compared with control group. The results are shown in Table.5.17 and 5.18.
6.3.3 Anti-nociceptive activity

The anti-nociceptive activity was performed for the crude leaves extract of STR and HAKS using various model like chemical induced writhing, formalin induced pain response and hot plate models. Pain is an acute or potential damage caused to the tissue, may be associated to the emotion or disagreeable sensory \(^{175}\).

The Pain may be sensed due to the ischemia, burn, infection, inflammation, and tissue necrosis. Inflammation of mucosa may cause the pain symptoms in the GIT (stomach and intestines) due to the muscle spasm or swelling. Pain may be off sudden and short-term due to the reflex removal\(^ {176}\). The pain is sensed by the peripheral fibers which conduct the pain signal through nerve. The higher or lower threshold of pain is caused by the nociceptors either by type I or type II A\(\delta\) fibers.

6.3.3.1. Central analgesic effect of STR and HAKS crude leaves extracts by Eddy’s hot plate.

Numerous natural products which possess digestive, neuroprotective, hepatoprotective anti-necrotic and anti-inflammatory properties have recently been discovered to have a potent antioxidant and/or radical-scavenging properties as part of their activity\(^ {177}\). Free radicals are dynamic mediators that exaggerate or tolerate inflammatory developments and, thus, their counteraction by
antioxidants and radical scavengers can diminish inflammation. Numerous flavonoids isolated from medicinal plants have been discovered to have substantial anti-nociceptive (analgesic) and anti-inflammatory properties. Therefore, it is possible that both the analgesic and anti-inflammatory activity is a mutual property of numerous flavonoid, terpenoids, phenols and sterols\textsuperscript{178}.

In hot plate method, the results showed that the ME-STR, HAE-STR, ME-HAKS and EAE-HAKS demonstrated significant analgesic activity in mice at a dose of 100, 200, and 400 mg/kg b.w. diclofenac sodium as positive control.

Experimental investigation showed that methanolic extracts of both plants possess potent analgesic activity at dose of 400 and 200 mg/kg (P≤0.001, P≤0.01). HAE-STR in the concentration of 400mg/kg (P≤0.05) and EAE-HAKS showed a potent activity at 400mg with (P≤0.01). This proved that both these plants have significant effect central analgesic activity when compared with positive control (diclofenac sodium 30mg/kg) with P<0.001. The results are shown in Table. 5.19 and 5.20.

6.3.3.2. Peripheral analgesic effect of STR and HAKS crude extracts on acetic acid-induced writhing test in mice

Analgesic action in mice, by inhibiting the acetic acid-induced writhing, is a model of instinctual pain\textsuperscript{179}. The writhing response of
the mouse to an intraperitoneal injection of toxic chemical is used to screen both peripheral and central analgesic activity. Acetic acid causes pain by redemptive endogenous materials and numerous chemical substances that stimulate pain nerve endings\textsuperscript{180}. Inhibition of peripheral tissues by cyclooxygenase enzyme thus, interfering with the mechanism of transduction in primary afferent nociceptors by obstructive the effect or the synthesis and/or release of inflammatory mediators\textsuperscript{181}.

All the extracts showed dose dependent analgesic activity. Among the extracts screened methanolic extract of both the plants were found to possess potent activity. This clearly proves that these plants possess analgesic activity. Since it was found that methanolic extracts have potent activity which clearly indicates the polar compounds like poly phenolic compounds triterpenoids glycosides may be responsible for the possible activity. The results are shown in Table 5.21 and 5.22.

6.3.3.3. Anti- nociceptive activity of STR and HAKS extracts on formalin induced pain in rat

The formalin induced nociception, work for both central and peripheral systems and studied by early and later or last phase\textsuperscript{182}. The early phase shows the pain related to non-inflammatory mediated pain, and the later stage the pain is reflected by the inflammation causative bio-processes. In the earlier phase central effect is mediated by direct activation of C fibers present in the nociceptors.
In later stage stimulation causes the release of substance P from the nerve ending which is related with chemical neurotransmitters like serotonin, prostaglandins, histamine and bradykinin mainly influenced by central effect. This is confirmed when the formalin induced pain was arrested by morphine in both the phases but the NSAIDs (aspirin) have only shown the inhibition of the pain in the last phase.

The reduction in licking of paw was seen for the methanolic crude extracts of both the plants at 400mg/kg.b.w at early and later phases.

EAE-HAKS produced a moderate response at the highest concentration while HAE-STR was not so effective in inhibition of pain when compared to the other extracts. The results are shown in Table. 5.23.

### 6.3.3.4. Antipyretic activity for the crude extracts of HAKS

Pyrexia (fever) is triggered on impact of infection, malignancy or other ailments. Any infection will damage the tissue and initiates production of numerous chemicals which form the pro-inflammation mediators. These mediators stimulate the biosynthesis of PGE$_2$. This triggers the hypothalamus to elevate the body temperature. Increased body temperature is reduced by regulating the nerve feedback mechanism by dilation of blood vessels and increasing the sweating.
Yeast-induced hyperpyrexia is a model for pathogenic fever. Thus, induction of the fever by the yeast triggers the production of the prostaglandins which stimulates the release of the phagocytic endogenous substance pyrogen which gets circulated in the blood and act on thermoregulatory center of hypothalamus by (cAMP- secondary messenger-mediated) mechanism\textsuperscript{185}.

Methanolic extract of HAKS showed dose dependent antipyretic activity at 200 and 400mg/kg. b.w. with the P value ≤ 0.01, when compared with acetyl salicylic acid (ASA), but the ethyl acetate extract did to show any significant reduction of pyrexia when compared with reference standard.

Past literature suggested that the antipyretic activity of ASA (acetyl salicylic acid) was due to inhibition of PGE\textsubscript{2}. This reduces of prostanoid material in pyrogen-sensitive areas of the brain \textsuperscript{186}. Hence the methanolic extract (ME-HAKS) might be inhibiting the synthesis of prostaglandin pathway similar to that of aspirin. The results are shown in Table. 5.24.

6.3.3.5. \textit{In-vitro} Anthelmintic activity screening for crude leaves extracts of STR and HAKS.

\textit{In-vitro} assessment of anthelmintic activity was carried our using Indian earthworm, tannins (polyphenolic) have been scientifically confirmed to possess the anthelmintic activities\textsuperscript{187}, and reports were documented for certain synthetic phenolic derivatives possessed anthelmintic activity by restricts the energy of production
in the helminth parasites by disconnection the oxidative phosphorylation. *In-vitro* anthelmintic results have shown that the major effect of albendazole on the worm to cause a flaccid paralysis that result in dismissal of the worm by peristalsis movement.

Albendazole works by accumulating the chloride ion in muscle membrane of worm to produces hyperpolarisation and reduced nervousness that leads to muscle relaxation and flaccid paralysis. From the result it is evident that various concentration (50, 100, 200, and 400 µg/ml) of the leaves crude extract of STR and HAKS showed paralytic and death time in concentration-dependence. Paralytic effect (P) was witnessed for crude extract of ME-STR, ME-HAKS, and EAE-HAKS (17.467± 1.525, 12.267 ± 1.707 and 15.450± 1.981) showed potent activity and HAE-STR (34.733 ± 1.519) showed a mild effect when compared with reference standard albendazole. The death time (D), were faster for the methanolic extract of both the plants 15.93 ± 3.18 (ME-HAKS) and 20.53 ± 1.14 (ME-STR), for ethyl acetate extract 23.05 ± 1.66 (EAE-HAKS) extract respectively.

All results was statistically analysed using student ‘t’ test one-way ANOVA, the P ≤ (0.0001) were significant when compared with standard group. The results are shown in Table. 5.25.