STUDIES ON *Sapindus mukorossii*
SAPONIN (REETHA SAPONIN)
2.1. Introduction:

India and other countries across the globe are facing one major problem—Rapid growth in population. As the number of people increase, it adds to the existing stress on available resources of food, water, house and health. The government and other agencies looking after the needs of the common man are already under pressure for providing basic necessities to one and all. Thus health and family planning has been adopted as the main agenda in many countries, especially India. According to the 9th five year plan given by the government of India - The task ahead of the country during the next decade is to bring about a convergence of all these favorable factors so that the twin goals of small healthy family and sustainable population growth are achieved within a decade. In view of the serious implications of the slow decline in the birth rate, efforts should be made to meet all the felt needs for contraception and achieve a more rapid decline in birth rates [1].

The discovery of oral contraceptive steroids and the use of I.U.C.D. in the late fifties have been important developments in contraceptive research. Though highly effective, the adverse side effects associated with their use - such as the risk of cardiovascular disorders like strokes or cancer or excessive menstrual blood loss or pelvic inflammatory diseases have limited their use. [2, 3] Barrier methods, like the use of condom, diaphragm or vaginal sponge are associated with disposal problems as well as chance of failure, due to improper use or rupture. Barrier contraceptives containing spermicides have the potential to be a valuable addition to the “menu” of choices available to women to take control of their reproductive activity. [2].
Vaginal contraceptives incorporating spermicides, if properly formulated and used, can be highly effective, since they are safe, free from systemic side effects and do not require any medical assistance or prescription. Therefore, there is an urgent need to develop new and effective, safe and cheap local contraceptives, particularly spermicides, which can also be effective in the control of sexually transmitted diseases like gonorrhea, syphilis and even AIDS.

**Requisites for an ideal spermicide:**

- Immediate death of spermatozoa on coming in contact with it.
- Aesthetic acceptability as a contraceptive method.
- Absence of toxic or irritant effect on the vagina.
- Easy to use
- Low cost.

During sexual intercourse, some of the ejaculated spermatozoa are often forced directly into the cervix, whence they rapidly move into the uterus. These spermatozoa are commonly those that will fertilize the egg. Therefore, it is essential for any spermicide not only to be distributed evenly over the vaginal epithelium but also have the capacity to diffuse into the cervical canal, possibly even altering the physico-chemical properties of the cervical mucus to render it “hostile” to penetrating spermatozoa.

**Status of the spermicides:**

Till the early seventies, there were more than 150 different formulations like jellies, creams, suppositories tablets etc., which were used all over the world. These were used by an estimated 10 million women’s, in more than seventy country all over
Studies on *Sapindus mukorosii* saponin (*reetha saponin*)

the world, but their use suffered a setback due to the discovery of oral contraceptive steroidal pills. However, the advantage of vaginal contraceptives incorporating spermicides lies in their positive value in protecting against infection particularly the fact that they can inhibit the growth of HIV, *Neisseria, Treponema, Gonorrhea* and even vaginal herpes virus.

Mode of Action of Spermicides:

The exact mode of action of several substances used as spermicides is not known. According to Mann (1964), spermatozoa are immobilised by both spermiostatic and spermicidal material and classified in four groups.

1. **Electrolytes:**

   The electrolytes disrupt the spermatozoa by creating osmotic imbalance. They have been found to be of little use as active ingredients in commercial spermicidal preparations as they are active at very high concentrations e.g. sodium chloride, potassium chloride etc.

2. **Enzyme inhibitors:**

   The second group includes enzymes inhibitors like cyanides, azides, fluorides, iodobenzoates, chloromercurobenzoates, iodoso-benzoate, hydrogen peroxide, 2:4-dinitrophenol etc. These compounds act on the various enzymes which play an important role in maintaining vital activity of spermatozoa, and stop specific functions of different enzymes.

3. **Sulphydryl binding substances:**

   The sulphydryl-binding agents are another group of substances, which act by combination with the sulfur-hydrogen bonds within the spermatozoa, thereby
Studies on Sapindus mukorossii saponin (*reetha saponin*) disturbing their metabolic activities. Most of the organometallic compounds, hydrogen peroxide, various quinones, copper, mercury and lead salts as well as arsenities and selenites are included in the class of sulphydryl binding agents.

4. Surface active agents (Surfactants):

The last and most important group is surface-active agents. Most of the present day spermicidal preparations available commercially contain one or more of them as active ingredient. The non-ionic group of surface-active agents is usually water-soluble and has a strong hydrophilic grouping e.g. Nonyl-9 (figure-1), Menfegol etc.

\[
\text{Fig 1 - Nonoxynol-9}
\]

The mode of action of these agents may be explained in two ways:

a) They reduce the surface tension at the cell surface and thus directly kill the spermatozoa by endosmosis. They also inhibit the oxygen uptake and fructolysis, and damage the plasma membrane of sperm.

b) Alternatively, their action may be more direct in that the molecules of these compounds may align themselves around the spermatozoa with the hydrophobic end of the molecules attached to the lipid layer of the surface and the hydrophilic end free in the surrounding medium. According to the Mann (1974) surface-active
agents are themselves neutralized by their inactivation of spermatozoa, which adds weight to the hypothesis involving such a surface attachment.

Commercially available vaginal contraceptives in use today are spermicidal agent, which rely upon surfactants such as their active ingredient [4,5]. These surfactants exert their effect by destroying sperm cell membrane resulting in rapid immobilization and cell death [6,7].

**Spermicidal Activity:**

The human semen is more resistant to spermicides than that of animals, [8] therefore for the in vitro testing of intravaginal spermicides some guidelines have been prescribed by Evaluation sub committee of the International Planned Parenthood Federation (I.P.P.F) in 1962.

**Testing of spermicides:**

Among several tests known for testing the activity of spermicides, two of the most widely used in vitro tests are the I.P.P.F agreed test for total spermicidal power and the Sander-Cramer Test [9].

**Formulations of spermicides:**

Different formulations of spermicides already developed are vaginal foams, contraceptive creams, sponges, jellies, cervical films, medicated diaphragms, vaginal suppositories, foam tablets, and medicated condoms.

Vaginal spermicidal foams- e.g. Delfen foam, Emko, Patentex, Profam etc.

Vaginal contraceptive creams- e.g. Conceptrol, Delfen, Flavocept etc.

Vaginal contraceptive jellies- e.g. Agena, Confidol, Gelifax etc.
Studies on *Sapindus mukorosii* saponin (*reetha saponin*)

Vaginal suppositories- e.g. Lorophyn, Norforms, Orthoforms, Rendells and Semicid etc.

The foam tablets, e.g. Today, Antibion, Neo Sampoon, Semori, Speton etc.

The contraceptive sponge **Today** sponge.

**Spermicides from Traditional Sources and Plants:**

Due to increase in interest of herbal-based medication many workers have analysed various plants and their extracts as possible spermicides [10,11]. Some have found flower extracts to be active [12] and some have recognised seed extract as active spermicide [13]. One of the oldest known efficacious plants was called silphium; it was apparently used to extinction by the third or fourth century AD. Hippocrates mentioned the use of Queen Anne's Lace (Wild Carrot), an inhibitor of progesterone production and fetal growth - the seeds of which are evidently still in use today (e.g., in parts of the rural United States). Other plants/fruits of alleged use are pennyroyal (which is toxic), asafoetida, artemisia, myrrh, rue, willow, date palm, pomegranate, cabbage, juniper, pine, onions, and acacia gum. The main problem with natural plant-based contraceptives has been inconsistency of action.

Research from India, China, and other countries have increased knowledge about the spermicidal characteristics of plant-based compounds such as Neem (*Melia azadirachta* or *M. indica*) and many others. Extracts of Neem and rock salts suspended in oil have been reported to kill human spermatozoa. Neem contains nimbin (Figure-2) which on hydrolysis gives nimbic acid and sodium nimbinate has been reported to exert spermicidal action at 0.5% concentration *in vivo* against rat's sperm [14]. The volatile, odorous fraction of neem oil coded as NIM-76 obtained by
Studies on Sapindus mukorosii saponin (reetha saponin) steam distillation was investigated for in vitro spermicidal activity [15]. The data showed that the minimum concentration, which inhibited spermatozoa motility, was 0.25 mg/ml for rat and 25 mg/ml for human spermatozoa. The effect of the drug on spermatozoa motility was found to be dose dependent. The activity of this drug was not altered in the presence of vaginal or cervical mucus. Intravaginal application of NIM-76 in rabbits showed no irritation to the vaginal mucous. Researchers in Reproductive Health are evaluating "Praneem polyherbal" cream and suppositories composed of three active ingredients: Neem seed extract; soapnut extract (Sapindus mukorosii); and quinine hydrochloride (for anti-HIV action). Praneem polyherbal cream has exhibited high spermicidal efficacy in animal studies and is being evaluated as a treatment for vaginal infection [16].

The aqueous extracts of plant Sapindus mukorosii Merr. also exhibited potent spermicidal action due to the presence of oleanolic acid (figure-2) [17]. Saponins isolated from other plants, which showed spermicidal action, include Bolbostemma paniculatum, [18], Ardisia nerifolia, Madhuca butyracea, Pittosporum nilgherense, Polemonium coeruleum, Schefflera capitata, and Trigonella foenum-graceum [19].
Studies on *Sapindus mukorosii* saponin (*reetha saponin*)

Other spermicidal agents isolated from natural sources include Solasodine from *Solanum kanisinum* (figure-3), *Brevipenin* from *Eupatorium brevipes* (figure-4) [20], Seminin [21] and Lebbekanim. [22].

Other plant extracts being investigated as possible spermicides with antimicrobial/antiviral properties include papain from papaya & gossypol from cotton seed [23], zinc acetate & lyophilized aloe barbadensis [24], saponins from *Phytolacca icosandra* [25] and, seed oil of *Pongamia glabra* [26].

*Abd. Elbary A & Nour SA* [27] studied certain plant saponins and then correlated the spermicidal activity exhibited by them to the hemolytic index. Whereas others have worked on evaluating spermicidal activity of plant saponins as such [28].

*Khanna et.al.* [17] screened the medicinal plants found in the Indian subcontinent for their spermicidal activity and were able to recognise the few plants which were most effective. *Kamboj & Dhawan* [29] have evaluated Indian medicinal plants for fertility regulation and spermicidal activity. *Khanna et.al.* [30] have also worked on saponins isolated from Indian plants as potential spermicidal agents.

Among the plants screened by them the most active spermicide was found to be the fruit extract of *Sapindus saponin* or reetha which was then taken up for further development at our institute. Spermicidal activity was found to be associated with the
Studies on *Sapindus mukorosii* saponin (*reetha saponin*)

β-amyрин C-28 carboxylic acid type of sapogenins, such as hederagenin, oleonolic and basic acids linked to a particular sequence of sugar moieties (figure-5 & 6)[31].

**Figure-5 structure of sapogenins like Bayogenin and Hederagenin.**

![Bayogenin (III)](image1)

**(2β-3β-form of 2,3,23-trihydroxy-12-oleanen-28-oic acid)**

![Hederagenin (IV)](image2)

**(3β-23-dihydroxy-12-oleanen-28-oic acid)**

The *Reetha* plant *Sapindus mukorosii* is found across the Indian subcontinent. It has been used in the Indian household since long time back as a cleansing agent for washing clothes and utensils, and bathing. Its fruit contains various saponins, which are mainly responsible for its cleansing properties.

The dried fruit is soaked and boiled in water to dissolve saponins that give a froth, which helps to clean by lowering the surface tension of water. At CDRI, the active extract from the fruit of *Sapindus saponin* has been tested and its formulation has been developed in form of a cream. Sapindus or *reetha* saponin is the n-butanol soluble fraction obtained from the ethanolic extract of the fruit pericarp of the plant *Sapindus mukorosii* [29, 31,32].

The saponins isolated from *Sapindus mukorosii* [33,34] were taken for further drug development at CDRI, Lucknow. For this, they were incorporated in a cream.
Studies on *Sapindus mukorosii* saponin (*reetha saponin*) referred to as *Consap cream*. Phase III multi-centric clinical trials are complete in seven State Medical Colleges and three Family Welfare Centres. Out of the 385 women enrolled, 254 have used Consap creams for more than six months while 131 have used the cream for less than six months. The cream has been found safe and efficacious, and permission for marketing from the Drug Controller General of India is received.

![Figure 6: Structure of Sapindoside -B and other Sapindus saponins identified.](image)

- R = Rhamnose (2→1) Arabinose. Sapindoside - A
- R = Xylose (3→1) Rhamnose (2→1) Arabinose, R' = H Sapindoside – B
- R = Arabinose (2→1) Rhamnose (3→1) Xylose(4→1) Glucose, R' = H Sapindoside – C
- R = Arabinose (2→1) Rhamnose (3→1) Xylose (4→1) Glucose [ (2→1) Glucose ] (5→1) Rhamnose, R' = H Sapindoside – D
- R = Xylose (3→1) Rhamnose (2→1) Rhamnose, R' = Acetyl Mukurozisaponin E1
- R = Xylose (3→1) Rhamnose (2→1) Arabinose, R' = Glucose (2→1) Glucose. Mukurozisaponin Y1

**Analysis of Saponins**- There are several methods used for saponin determination in plant materials. It is observed that the biological and spectrophotomeric methods which are being used for saponin determination provide, to a larger degree, valuable
Studies on Sapindus mukorossi saponin (*reetha saponin*)

results on saponin concentrations in plant material. However, one plant species may contain some saponins which can be determined with a biological test and others which cannot. That is why biological and colourimetric determinations do not provide accurate data and have to be recognized as approximate. Gas–liquid chromatography has limited application for determination since saponins are quite big molecules and are not volatile compounds. Thus, there are only few applications of GC for determination of intact saponins. The method has been used for determination of TMS, acetyl or methyl derivatives of an aglycones released during saponin hydrolysis. However, structurally different saponins show different rates of hydrolysis and precise optimization of hydrolysis conditions is essential. Besides, during hydrolysis a number of artifacts can be formed which can influence the final results.

Thin-layer chromatography on normal and reversed-phases (TLC, HPTLC, and 2D-TLC) is being used for routine determination of saponins in plant material. High performance liquid chromatography technique for saponin determination is the most-widely used method for this group of compounds. However, the lack of chromophores allowing detection in UV, limits the choice of gradient and detection method. The pre-column derivatisation with benzoyl chloride, coumarin or 4-bromophenacyl bromide has been used successfully in some cases allowing UV detection of separation. Standardisation and identification of the peaks in HPLC chromatograms has been based on comparison of the retention times with those observed for authentic standards. But new hyphenated techniques, combining HPLC with mass spectrometry and nuclear magnetic resonance are developing rapidly and allow on-line identification of separated saponins. Capillary electrophoresis has been applied for
Studies on Sapindus mukorosii saponin (*reetha saponin*)

Saponin determination only in a limited number of cases and this method is still being developed [35].

A colourimetric assay method using vanillin and sulfuric acid was developed earlier for the estimation of the total saponin content of the spermicidal *reetha saponin* [32]. In the recent years, chromatographic techniques like - HPLC, LC-MS and HPTLC have been used for fingerprinting and estimation of saponins from different plants [35,36,37,38]. Here we put forward two different methods for analysis of various sapindosides within the saponin mixture. We have developed and used HPLC and HPTLC methods for the fingerprinting and estimation of Sapindoside-B [I], one of the major active constituents, in bulk drug samples of *Sapindus saponin* and its formulation *consap cream* [32,33,34]. And then by using LC-MS technique identity of six sapindosides including Sapindoside-B was established in the *Sapindus saponin* mixture.
Studies on Sapindus mukorossii saponin (*reetha saponin*)

Experimental

2.2.1. Reagents and standards:

Standard *sapindus saponin* was provided by Chemical Technology Division, of this Institute. HPLC grade acetonitrile, methanol and chloroform were obtained from M/S E. Merck (India) Limited (Mumbai, India). Triple distilled water, from an all glass apparatus, was used as solvent. Other reagents like naphthorescorcinol, ethanol, H$_2$SO$_4$ (96%) used were of analytical grade. All glassware were washed with detergent, rinsed thoroughly with triple distilled water and dried prior to use.

2.2.2. Isolation and Purification of Sapindoside B [I]:

Pure Sapindoside-B was obtained by column chromatography of the *sapindus saponin* and the identity of Sapindoside–B [I] and other five sapindosides was established by Electro-spray Mass Spectra. To isolate pure sapindoside-B, chromatographic techniques-TLC & column chromatography using several eluent systems was tried. Initial attempts were unsuccessful as most of the saponins have very similar structure. Finally we arrived at the following method of separation which combines column chromatography and TLC.

About 5 gm of *Sapindus saponin* was loaded on a silica column (~250gms). The elution was done initially with chloroform followed by chloroform containing increasing proportions of methanol and water. Various fractions were collected and checked by TLC. The spots corresponding to fraction enriched with sapindoside B were mixed and concentrated to dryness under reduced pressure. This enriched fraction was again loaded on a silica column. The fractions were eluted with chloroform: methanol: water (89.5:10:0.5 - 84.5:15:0.5) and those corresponding to
Studies on Sapindus mukorosii saponin (*reetha saponin*)

Sapindoside-B were pooled, concentrated to dryness under reduced pressure. The MS of this purified fraction using ES-MS showed a major peak at 905 (M+Na), for sapindoside B [I] (Figure-7). Similarly fractions containing other sapindosides were collected, checked by TLC and their LC-MS data using ESI were generated.

Final purification of sapindoside-B was done by prep-TLC. The fraction having sapindoside-B was dissolved in methanol and loaded in form of a single band on the precoated silica gel TLC plates (20x20cm, 0.25mm, Merck). The plates were developed in chloroform: methanol: water (50:14:1) system and the region corresponding to sapindoside-B was scrapped off from the plates, extracted by mixture of methanol and chloroform (90:10, 5x10ml). The extracts were combined and evaporated to dryness under reduced pressure. The purity and identity of the solid obtained was checked by TLC, HPLC and ES-MS (Figure-7).

**2.2.3. Preparation of stock and working standard solution**

Stock standard solution containing 1 mg/ml of sapindoside B was prepared by dissolving 10 mg of purified sapindoside-B in 10 ml of methanol. The working standards for HPLC were prepared by serial dilution from the stock solution of sapindoside B accordingly in the range of 30 μg/ml to 200 μg/ml in methanol. Whereas for HPTLC the stock was used as such for spotting by automatic TLC sampler.

**2.2.4. Preparation of sample solution:**

The samples of the *Sapindus saponin* (25 mg) each from different batches were dissolved in 10 ml methanol and analysed. Also different batches of consap cream were taken for analysis.

[ 74 ]
2.2.5. Extraction of saponin from consap cream:

About 1 gm of the sample of the consap cream was suspended in 10-ml water (saturated with n-butanol). This solution was extracted with n-butanol (saturated with water, 3x10 ml). The organic layer was washed with water (saturated with n-butanol, 3x10 ml). The organic layer was freed of solvent under reduced pressure. The dried saponin mass so obtained was dissolved in 10-ml methanol and analysed.

2.2.6. Apparatus and chromatographic conditions for HPTLC:

The HPTLC system was equipped with auto TLC sampler–3 with air pressure injector (CAMAG), syringe injector with a 50 µl loop, twin chamber TLC developer and Camag Scanner ATS-3 with tungsten, mercury and deuterium as detector lamps. HPTLC separation was achieved on a silica gel pre-coated plate (20cmX20cm, silica gel 60F254) from Merck, for detection of the spots CAMAG TLC scanner was used. Data was acquired and processed using a Camag HPTLC software CATS - 4.

2.2.7. HPTLC method: Various spots of the standard sapindoside-B in the range of 5.0-220 µg and three spots of 100µl each of the sample were loaded on the silica plate. The plates were developed in the solvent system - chloroform: methanol: water:: 50:14:1. Chromatography was performed at 27 ± 3° C. The plates were removed, dried and the spots were visualized by post chromatography derivatisation with naphthorescorcinol reagent \[22\] \{naphthorescorcinol 20 mg in 25 ml ethanol + 0.4 ml H₂SO₄ (96%)\} and heating the plate at 100° C for 10 minutes. The blue coloured spots were scanned at 630 nm.
Studies on Sapindus mukorossi saponin (reetha saponin)

**Figure 7:** ES-MS spectra of Sapindoside -B.

ES-MS spectra of Sapindoside -B

*Sapindoside-B (M+Na)=905 by ES-MS*
Apparatus and chromatographic conditions for HPLC:

The HPLC workstation used was from Shimadzu, Japan; equipped with SCL-10A VP system controller, LC-10AT VP twin pump, SPD-10A VP UV-VIS detector, Rheodyne injector with 20 µl injection loop. The separation was achieved on a 5µ Lichrosphere C-18 (25 cm x 0.4 cm) Lichrocart reverse phase analytical column (from Merck). The data acquisition and processing was done by using Shimadzu HPLC software class-VP (V5.03).

The LC-MS experiment was performed on a MICROMASS QUATTRO II triple quadruple mass spectrometer. The ESI capillary was set at 3.5 kV and the cone voltage was 40V. The spectra was collected in 6s scans and the final prints were the averaged spectra of 6-8 scans. The column used was µ-bondapack C-18 (30 cm x 0.39 cm, Waters) reverse phase analytical column.

HPLC method: The separation of different saponins was achieved on C-18 (25 cm x 0.4 cm, 5µm) reverse phase column (E Merck) and also on C-18 (30 cm x 0.39, 6µ) reverse phase column (Waters). The solvent system was acetonitrile: water gradient starting with 90%water to 20% water in 30 minutes. The eluents were detected at 215 nm. The chromatography was performed at 27±3°C.

LC-MS of sapindosides in sapindus saponin: By the above described method of HPLC, using µ-bondapack C-18 column (Waters) and ESI MS, the LC-MS was performed for various enriched fractions of saponins obtained from column chromatography. The identity of saponins was established by Selected Ion Monitoring (SIM) of the Total Ion Current (TIC) at the required mass numbers.
Studies on Sapindus mukorossii saponin (reetha saponin)

**Accuracy and precision:** The accuracy of the method was calculated on the basis of the difference in the mean calculated and added concentrations and the precision was obtained by calculating the inter and intra day relative standard deviations (% R. S. D.) [40].

**Recovery:** Known amounts of sapindoside B was added to the mixed contents of the samples/cream and sapindoside B in the sample/cream was determined by interpolation on the corresponding calibration graphs.
Studies on *Sapindus mukorosii* saponin (*reetha saponin*)

**Result and Discussion**

The method discussed above provided good separation of individual constituents, suitable for fingerprinting of *Sapindus saponin*. Purification and estimation of sapindoside-B was also done by the method described above.

**Chromatography:** *Sapindus saponin* is a mixture of more than ten components. Most of them are structurally related as basic structure is the same and only the type and number of sugar moieties attached constitute the different saponins (Figure-6). Therefore proper resolution of the individual components for quantitation as well as estimation among most of the saponins becomes fairly difficult task.

Initial experiments using silica gel TLC plates and several solvent systems with different ratio of ethyl acetate, methanol, chloroform including buffers were tried. No proper separation of the individual components was obtained. Chloroform: methanol: water (50:14:1) gave the best separation of the spot corresponding to sapindoside-B from ten other prominent spots (Figure-8).

The purified fraction of sapindoside-B from column chromatography was confirmed by LC-MS. Further the acid hydrolysis of this purified fraction revealed the presence of three sugars xylose, rhamnose and arabinose.
Figure 8: The HPTLC separation of Sapindoside-B. and other constituents- (a) Spots (1-6) and (13-18) of Sapindoside-B concentration range 5μg -220μg; (b) Spots (7-8) of sample 1 (c) Spots (9-10) of sample 2 (d) Spots (11-12) of sample 3.
Studies on Sapindus mukorosii saponin (*reetha saponin*)

Figure-9  HPLC separation of Sapindosides in the Sapindus saponin mixture

For HPLC, a C-18 reverse phase analytical column (from Merck) and the acetonitrile-water gradient system gave proper separation of the compound. The HPLC method described provides a good separation of I from the other sapindosides (Figure-9 and 10).

**Fingerprinting of sapindus saponins:** For LC-MS, above described HPLC method using C-18 column from Waters and MS were used. We were able to recognise six saponins (Figure-6): sapindoside-A (M+Na=773) at 12.56 min, sapindoside-B (M+Na=905) at 11.6 min, sapindoside-C (M+Na=1067) at 5.79min, sapindoside-D (M+Na=1375) at 10.91min, mukorozisaponin-E1 (M+1=924) and mukurozisaponin-Y1 (M+1=1207) at 4.13min (figure-11)(Table-1).
Figure-10- HPLC Chromatogram of Sapindoside-B

Detector A - 1 (215nm)

Table 1: Finger-printing of Sapindosides in *Sapindus saponin*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Sapindosides</th>
<th>Mass</th>
<th>M + Sodium ion</th>
<th>Retention time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sapindoside-A</td>
<td>750</td>
<td>773</td>
<td>12.56</td>
</tr>
<tr>
<td>2</td>
<td>Sapindoside-B</td>
<td>882</td>
<td>905</td>
<td>11.6</td>
</tr>
<tr>
<td>3</td>
<td>Sapindoside-C</td>
<td>1044</td>
<td>1067</td>
<td>5.79</td>
</tr>
<tr>
<td>4</td>
<td>Sapindoside-D</td>
<td>1352</td>
<td>1375</td>
<td>10.91</td>
</tr>
<tr>
<td>5</td>
<td>Mukurozisaponin-E1</td>
<td>924</td>
<td>947</td>
<td>13.52</td>
</tr>
<tr>
<td>6</td>
<td>Mukurozisaponin-Y1</td>
<td>1206</td>
<td>1207(M+1)</td>
<td>4.13</td>
</tr>
</tbody>
</table>
Studies on *Sapindus mukorossii* saponin (*reetha saponin*)

**Figure 11:** Selected ion monitoring of sapindosides by LC-MS

- (a) mukurozisaponin-Y1
- (b) sapindoside-C
- (c) mukurozisaponin-E1
- (d) sapindoside-B
- (e) sapindoside-A
- (f) sapindoside-D
Studies on Sapindus mukorosii saponin (*reetha saponin*)

**Selectivity and specificity:** The retention factor on HPTLC of I was 0.48, and other sapindosides were found to be, 0.19, 0.26, 0.41, 0.59, 0.67, 0.75, 0.83, 0.90, 0.94 (Figure-8). Based on the noise to signal ratio of 3, the detection limit in HPTLC was 5 μg, however the limit of quantitation was set at 10 μg.

The retention time on HPLC of I was found to be about 15 min (Figure-10). Under the chromatographic conditions mentioned above, peaks corresponding to other constituents did not interfere with the peak of I (Figure-9). The detection limit in this case was 10 μg and the limit of quantitation was set at 30μg.

Both the methods provided adequate sensitivity for the determination of I in bulk substance and dosage forms.

**Linearity and reproducibility:** In HPTLC and HPLC, external standardization by peak area was used for the quantitative determination of I.

**Figure 12:** Calibration graph of Sapindoside – B using HPTLC.
Studies on *Sapindus mukorossii* saponin (*reetha saponin*)

In HPTLC the calibration curves were linear in the range of 11 µg to 220 µg with $r=0.99948$ (Figure-12), while in HPLC the calibration curve was linear in the range of 30 µg to 200 µg with $r=0.9912$ (Figure-13).

Table 2: Inter and intra assay variations by HPTLC.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Conc. taken</th>
<th><strong>Intra-assay variation</strong></th>
<th><strong>Inter-assay variation</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean conc. Found SD.</td>
<td>%DFA</td>
</tr>
<tr>
<td>1.</td>
<td>5.5</td>
<td>2.6 ±0.4</td>
<td>-52.72</td>
</tr>
<tr>
<td>2.</td>
<td>11</td>
<td>12.04 ±0.83</td>
<td>9.45</td>
</tr>
<tr>
<td>3.</td>
<td>27.5</td>
<td>27.71 ±0.61</td>
<td>0.76</td>
</tr>
<tr>
<td>4.</td>
<td>55</td>
<td>55.27 ±5.21</td>
<td>0.49</td>
</tr>
<tr>
<td>5.</td>
<td>110</td>
<td>112.18 ±2.08</td>
<td>1.98</td>
</tr>
<tr>
<td>6.</td>
<td>220</td>
<td>216.28 ±1.4</td>
<td>-1.69</td>
</tr>
</tbody>
</table>

Figure 13: Calibration graph of Sapindoside – B using HPLC
Studies on Sapindus mukorosii saponin (*reetha saponin*)

Table 3: Inter and intra assay variations by HPLC

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Conc. Taken</th>
<th>Intra-assay variation</th>
<th>Inter-assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean conc. Found ±SD</td>
<td>%DFA</td>
</tr>
<tr>
<td>1.</td>
<td>30.26</td>
<td>30.94±1.12</td>
<td>2.26</td>
</tr>
<tr>
<td>2.</td>
<td>60.52</td>
<td>60.3±0.008</td>
<td>-0.36</td>
</tr>
<tr>
<td>3.</td>
<td>90.78</td>
<td>88.89±2.51</td>
<td>-2.08</td>
</tr>
<tr>
<td>4.</td>
<td>121</td>
<td>122.29±0.45</td>
<td>1.07</td>
</tr>
<tr>
<td>5.</td>
<td>181.5</td>
<td>176.55±1.41</td>
<td>-2.73</td>
</tr>
<tr>
<td>6.</td>
<td>210.04</td>
<td>211.69±0.72</td>
<td>0.78</td>
</tr>
</tbody>
</table>

The reproducibility and accuracy of both the methods were determined by intra- and inter day assay variation (Table 2-3). Reproducibility and accuracy of the method were within the acceptable limit [40].

**Recovery:** The recovery from the consap cream was checked by extraction of saponins from the cream and estimation by the described method. It was found that the amount of saponins recovered from the cream was more than 90%.

**Application of method in pharmaceutical analysis:** Several samples of bulk preparation and consap cream for vaginal irritation and efficacy studies were analyzed by the present method. Estimation of sapindoside-B in the bulk samples for formulation development was also done (table-4). Estimation of *Sapindus saponin* and Sapindoside-B in consap cream after storage was also checked using this method (table-5).
Studies on Sapindus mukorossi saponin (*reetha saponin*)

Table 4: Estimation of Sapindoside-B in *Sapindus saponin* bulk drug samples

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Year of manufacture</th>
<th>% Sapindoside B found</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-1997</td>
<td>1997</td>
<td>5.4</td>
</tr>
<tr>
<td>4-1998</td>
<td>1998</td>
<td>7.2</td>
</tr>
<tr>
<td>2-2002</td>
<td>2002</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Table 5: Estimation of Sapindoside-B in formulation *consap cream* after storage.

<table>
<thead>
<tr>
<th>Year of preparation</th>
<th>Label claim (% w/w)</th>
<th>Saponin extractive found (% w/w)</th>
<th>Sapindoside-B % estimated in extracted saponin</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
<td>2.5</td>
<td>0.97</td>
<td>2.22</td>
</tr>
<tr>
<td>95</td>
<td>2.5</td>
<td>1.38</td>
<td>6.33</td>
</tr>
<tr>
<td>97</td>
<td>2.5</td>
<td>2.39</td>
<td>8.16</td>
</tr>
</tbody>
</table>
Studies on Sapindus mukorosii saponin (*reetha saponin*)

**Conclusion**

The HPTLC method so developed is simple and can be easily used. Further due to post-chromatographic derivatisation using naphthorescorcinol reagent significant improvement in the sensitivity and limit of quantitation of Sapindoside-B was achieved. By this method proper resolution between *sapindus saponins* was also obtained.

HPLC method also provides good resolution between the sapindus saponins. The method developed is sensitive and can be used for the analysis of bulk samples of *sapindus saponins* and *consap cream*. Further, using LC-MS, fingerprinting of *sapindus saponin* can be done.

Both the methods provide good separation and resolution among various saponins. Both can be easily used for routine estimation of sapindoside-B in bulk drug samples of *sapindus saponin* and *consap cream* and chemical fingerprinting of *sapindus saponin*. 
Studies on *Sapindus mukorosii* saponin (*reetha saponin*)

REFERENCES

1. 9th five-year plan for Human and social welfare ministry, Government of India.


Studies on *Sapindus mukorossi* saponin (*reetha saponin*)


33. HS Garg *et al.*, *Indian patent No*. 141240.

34. NM Khanna *et al.*, *Indian patent No*. 150335.
Studies on Sapindus mukorosii saponin (reetha saponin)


39. Concise practical book of thin layer chromatography -Author- Prof. Dr. Ljubomir Kraus, DESAGA GmbH, Heidelberg.