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Research Article

**THIN LAYER CHROMATOGRAPHY - AS A TOOL FOR
STANDARDIZATION OF AYURVEDIC MEDICINE, *TRIPHALA
CHURNA***

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Running title: TLC as Medicinal Standardization Tool for *Triphala churna*

Abstract:

Ayurvedic and other traditional forms of medicines require standardization to bring them at par with modern molecular medicine. The present study is to use thin layer chromatographic technique to understand the presence and variation of four important secondary metabolites namely, steroids, terpenoids, flavonoids and alkaloids in the Ayurvedic preparation, Triphala churna and its constituent ingredients, namely, Terminalia bellerica, terminalia chebula and Embelica officinalis. The TLC profiles of steroids, terpenoids, flavonoids and alkaloids indicated different behaviour for only rinds, only seeds, their respective mixtures, whole fruits separately and their mixtures. The results were compared with standards of each metabolite and also with market samples. It was observed that alkaloids were almost similar in all the samples indicating their predominance in Triphala ingredients as well as those of mixtures. Embelica officinalis was having more dominating and masking effect over the other two constituents for all the four metabolites studied. It was also clear that most of the market samples indicated the presence of whole fruits as their raw materials instead of the rinds alone, which needs to be addressed. From the study it is clear that this methodology can be used as a cheap tool to estimate the presence of the constituents in each sample and suggest this easy method for quality analysis of the samples before they come to market.

Key Words: TLC, Triphala churna, Terminalia bellerica, Terminalia chebula, Embelica officinalis, Rf, standardization, Prednisolone, Quercetine, Caffine, Retinol.

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INTRODUCTION:

The role for complementary and alternative medicinal practice is gaining momentum in the recent past. Ayurveda, Sidhha, Unani, Yoga, Homeopathy, Chinese and other forms of traditional medicinal practices are being exploited to understand their beneficial effects as compared to the modern molecular medicinal practice which is known for its short and long term side effects. Ethno botanically many native medicines are being used since time immemorial but their efficacy, validity and effectiveness is being discovered only in the recent past. The scientific evaluation and standardization of the traditional medicines is a huge challenge since there a dearth of information about the efficacy of these forms of medicines in the light of modern medicinal parameters like, pharmacological, pharmacokinetic, teratological, long and short term side effects, molecular mechanism of action etc. Of late some encouraging reports in this regards are accumulating. Focus is being given to standardize these medicines at each level, such as at procuring, processing, storing, transporting, dispensing etc. The use of modern techniques such as phytochemical analysis, Thin Layer Chromatography, High Power Thin Layer Chromatography, High Power Liquid Chromatography, Nuclear magnetic Resonance, Infra red and Ultra violet Spectroscopy, Gas Chromatography Mass Spectroscopy, Liquid Chromatography mass spectroscopy, X Ray Diffraction etc. are being used to understand and analyze the chemical mechanism of the medicines. These studies could help in proving the scientific efficacy of such medicines [1-37].

The present study deals with the Thin Layer Chromatographic analysis of on Ayurvedic medicine, *Triphala Churna*, to screen the presence of various bio-molecules of medicinal importance, present in it. Ayurvedic formulation *Triphala* is a polyherbal medicine made of a mixture of powders of three fruits, *Terminalia bellerica*, *Terminalia chebula* and *Embelica officinalis* in equal proportion. This Ayurvedic medicine is used by all age groups as colon tonic, eye rejuvenator, antibacterial and as blood purifier. *Triphala* is rich source of antioxidants such as gallic acid, beta- sitosterol and flavonoids etc. which could play important roles in cure of many diseases such as anemia, constipation, fever, inflammation, infections, gastrointestinal heat, stress reduction, diabetes, cardio vascular disease etc. *Triphala rasayana* is the liquid form of *Triphala*, the GC MS analysis of which indicated the presence of some important compounds namely, β -Sitosterol, Oleic Acid, 2-[4-methyl-6-(2,6,6-trimethylcyclohex-

1-enyl) hexa-1,3,5,1-Monolinoleoylglycerol trimethylsilyl ether and 1H-2,8a-ethanocyclopenta[a]cyclopropa[e]cyclodecen-11-one, which have various medicinal properties which augur well with the similar properties of *Triphala rasayana* (Muthiah *et al*, 2017) [38].

The three constituents of *Triphala*, namely, *Terminalia chebula*, *Terminalia bellerica* and *Embelica officinalis* are age old medicinal fruits used for their various clinical values which are briefly mentioned hereunder.

TERMINALIA CHEBULA

Terminalia chebula has lots of pharmacological activity such as anti carcinogenic activity, chemopreventive activity, anti-oxidant activity, radio protective and free radical activity, cardio protective activity, anti -bacterial, anti viral anti fungal, anti arthritis, anti inflammatory, anti anaphylactic activity, hypo cholesterolmic, hypolipidemic, anti spasmodic activity, anti allergic etc.

Fruits also have lots of beneficial effect in bleeding gums, ophthalmia, dental caries and analgesic, anti inflammatory and also used for chronic diarrhea, chronic cough, renal calculi, allergies, constipations, irregular fever, ulcers, heart disease, diabetes, digestive disease, hemorrhoids, asthma, hiccough, vomiting, dysentery, sore- throat, diarrhea, ulcers, gout and other heart related disease. This plant also have multiple activities in pharmaceuticals and the field of medicine such as anti-diabetic, anti inflammatory, anti-proliferative, gastrointestinal motility, wound healing, anti mutagenic, antimicrobial. The dried fruits also having several phyto-constitutes including steroid, flavonoid, alkaloid, terpenoid, chebulagic acid, chebulanin, gallic acid, neochebulinic acids, casuarinin, tannins, terchebulin, corilagin, terflavin, punicalagin, beta-sitosterols, amino acids, maslinic acid, flavonol, glycosides and also having fatty acids like oleic acid, linoleic acid and palmitic acid (Bag *et al*, 2013) [39]

TERMINALIA BELLERICA

Terminalia bellerica contain several phyto-constituents, which are found in seeds, leaves, peel of fruits and in whole plants; these phyto-chemicals includes flavones, steroids, tannin, ellagic acids, gallete, gallic acids, glycosides, terpenoid, saponin. *Terminalia bellerica* shows several pharmacological properties and functions these are anti-diabetic, anti-fertility, anti-fungal, analgesic, anti-cancer activity, anti-diarrheal activity, anti- hypertensive effect, anti mutagenic effect, anti-oxidant, anti-secretory, anti-spasmodic, antithrombotic, anti ulcer activity,

immune modulator activity, gastro intestinal, spleen, dysentery, cough and also wound healing. (Saraswathi *et al*, 2012) [40]

EMBELICA OFFICINALIS

Amla is used for treatment of high blood cholesterol, liver toxin, age related kidney disorder. The fruits are used as a diuretic, laxative and in case of dysentery and diabetes dried fruits are used and also for jaundice, anemia, the oil of the Amla promotes the hair growth. Fruits are very rich in vitamin-C and have lots of anti oxidant. *Embelica officinalis* contains protein, fat, fibre, nicotinic acids, vitamin-B₃, calcium, carbohydrate, phosphorous and iron it also contain several phyto-chemicals such as- tannins, alkaloid, terpenoid and juice of this fruits contain maximum amount of vitamin-c and other compounds such as gallic acids, ellagic acids, chebulicnic acids, corilagin. The various beneficial effects attributed to this fruit are its antioxidant activity, anti-pyretic, analgesic activity, anti-ulcer, hepato protective, anti-cancer, cardio protective, anti-tumor, cyto and gastro protective activity, as chelating agent, snake venom neutralizer, good memory enhancing activities. This is also used for cure various disease like nausea, constipation, diabetes, skin cancer, head ache, dental problem. (Bhide and Nitave, 2014; Dasaraju and Gottumukkala, 2014) [41, 42]

MATERIALS AND METHODS:

The study is aimed at TLC analysis of important secondary metabolites such as steroids, terpenoids, flavonoids and alkaloid of raw materials and final product of *Triphala churna*. The fruits were studied with seeds and without seeds to find the influence of the seeds on the medicine, since, only the rind of the fruit is supposed to be used to make *Triphala churna* and not the whole fruit as per Ayurvedic scriptures.



Figure 1a.

Figure 1b

Fig 1 a. Fruits of *Terminalia bellerica*, *Terminalia chebula* and *Embelica officinalis*.

Figure 1b. Powders of *Terminalia bellerica*, *Terminalia chebula* and *Embelica officinalis*.

Principle of chromatography

The principle of chromatography is adsorption and partition, while mixture of substance is allowed to pass through stationary and mobile phase, a compound become separated according to their R_f.

R_f = distance travelled by the sample salute /distance travelled by solvent front

Preparation of sample

The three ingredient fruits, namely, *Terminalia bellerica*, *Terminalia chebula* and *Embelica officinalis* were collected from standard Ayurvedic vendor at Chennai. They were thoroughly washed to remove superfluous dust and dried. The one batch, the three fruits were separately powdered and equal quantity of each powder was mixed to make a *Triphala churna*.

In the second batch, all constituent fruits were broken to get the fruit rind and the seeds. The fruit rinds and the seeds were ground separately. The three different rinds were powdered separately and then mixed in equal proportions and similarly, the three different seeds were powdered and then mixed in equal proportion. This was to prepare samples of *Triphala churna* without seeds and only with seeds, to find any variation expressed in these two samples. Thus three different samples TP (1:1:1, all three fruits), Cns (1:1:1 of three rinds), Csp (1:1:1 of three seeds). Additionally, to find any influence of each fruit on the customized sample, each constituent was added in additional quantity (i.e. CS+TC; CS+TB and CS+EO).

Solvent preparation: Different solvent systems were used for each phytochemical as mentioned below:

- Solvent system for steroid : Hexane : Ethyl acetate (1:1)
- solvent system for terpenoid : Hexane: Acetic acid (9:1)
- solvent system for flavonoid : Toluene :Acetic acid (9:2)
- solvent system for alkaloid : Ethyl acetate :Methanol: Water (12:35:1.5)

Developing chamber saturation

Before running the TLC, the saturation of the chamber is performed by pouring the separating solution in the chamber and to stand for 30-60 minutes with a lid over the top of the chamber so that it becomes saturated with mobile phase solvent.

TLC plate preparation and running

Silica Gel and distilled water mixed to get thick slurry, then placed and spread over the un reactive

career such a glass or plastic. Plates were dried. Plates were marked at bottom and top for spotting sample, to stop running of solvent beyond end of the slides marking done also at the top. Capillary tube dipped into respective test solution so that solution rise up in the tube. Prepared plate where briefly touched by the capillary tube at the start line so that

the test solution get absorbed. Plates were placed at the developing Chamber Containing solvent of interest after sometime solvent front reached at the top near the marked line. Plate were taken out and dried to evaporate the solvent at the stationary phase. Visualization of spots was carried out by putting the plates in iodine chamber.

RESULTS AND DISCUSSION:

Table 1 represents the rf values of customized, market and standard samples along with individual components and those of standards.

Table 1: Shows Rf value of customized, market and standard samples along with individual components and those of standards.

Sample	Steroid	Terpenoid	Flavonoid	Alkaloid
TC ns	0.2	0.3538	0.3384	1, 0.75381
TB ns	0.7692,0.2, 0.6923	0.3384	0.3076	1, 0.5384
EO ns	0.2, 0.2307,0.3538	0.3076, 0.3846, 0.6923	0.3384, 0.8923	0.3076, 1
(TCTBE O) or Cns	0.2, 0.6923, 0.4923	0.3384, 0.6923, 0.4	0.3384, 0.8923	0.4, 1
TC SP	0.4615	0.9393, 0.6153, 0.5076, 0.7692, 0.5692	1, 0.8461	0.2461,0.9538
TB SP	0.4615	0.9393,0.5076 0.6153,0.5692, 0.6461	1, 0.3384, 0.8461	0.2615, 0.9538
EO SP	0.2307, 0.7692	0.2461, 0.5692, 0.3076,0.6461	0.3538, 0.8461, 0.8769,0.9538	0.3538, 0.9692, 1, 0.2307
C SP	0.2307, 0.7230	0.2461, 0.6461, 0.5692, 0.3076	0.3538, 0.8461, 0.8769, 0.9538	0.2307, 0.3538, 0.9692, 1
T C	0.2923, 0.4923	0.3076, 0.3846	0.8769, 1	0.8307, 1
TB	0.2923, 0.4769	0.3076, 0.4	0.8615	0.8307, 1
EO	0.3692, 0.6307	0.4615, 0.5846,0.7076	0.5846, 0.8615, 1	0.8461, 0.9846
T. P (m)	0.2923	0.2307,0.2769,0.8153	0.446,0.984,1	0.7846, 1
TC+TB	0.3538, 0.5230	0.2307	0.3384, 0.3846	0.4615 ,0.5692, 0.7692, 1
TC+EO	0.4461	0.2461	0.3384, 0.3846	0.465, 0.5384, 0.7692, 1
TB+EO	0.2461, 0.2923, 0.4	0.2307	0.3846, 0.8461	0.3846, 0.5230, 0.9846
m+TC	0.2615, 0.2923, 0.3846	0.2307	0.3846, 0.8461	0.3846, 0.5692, 1
m+TB	0.3230, 0.4769	0.2307	0.3846, 0.8461	0.3846, 1
m+EO	0.3230, 0.4615, 0.8461	0.2461	0.2, 0.4, 0.8461	0.4, 1
MI	0.2307	0.2769	0.2923,0.8461	0.4923, 0.9846
M2	0.2307	0.2769	0.2923, 0.8461	0.4923,0.9846
M3	0.2615	0.3076,0.3538	0.2615, 0.8769	0.69230,1
M4	0.2461	0.3076, 0.3538	0.2769,0.8923	0.5230,1
M5	0.2307	0.7692	0.2923, 0.8769	0.9846
1 S—P	0.4, 0.49230			
2F—Q	-	-	0.507,0.846,1	-
3A—C	-	-	-	0.3692,1
4T—A	-	0.3076,0.3846,0.4615,0.837	-	-

NS =No Seed; SP = Seed Powder; Standards: P-Prednisolone; Q-Quercetine; C- Caffeine;

A-Retinol

Steroid TLC Profile Study. Figure 2, 3, 4 and 5 indicate the TLC profiles of Steroids of samples.

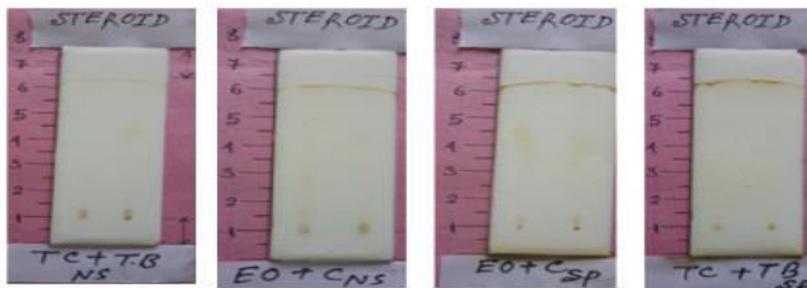


Figure 2. Steroid TLC profile of *T. chebula*, *T. bellerica* and *E. officinalis* rinds and seeds.

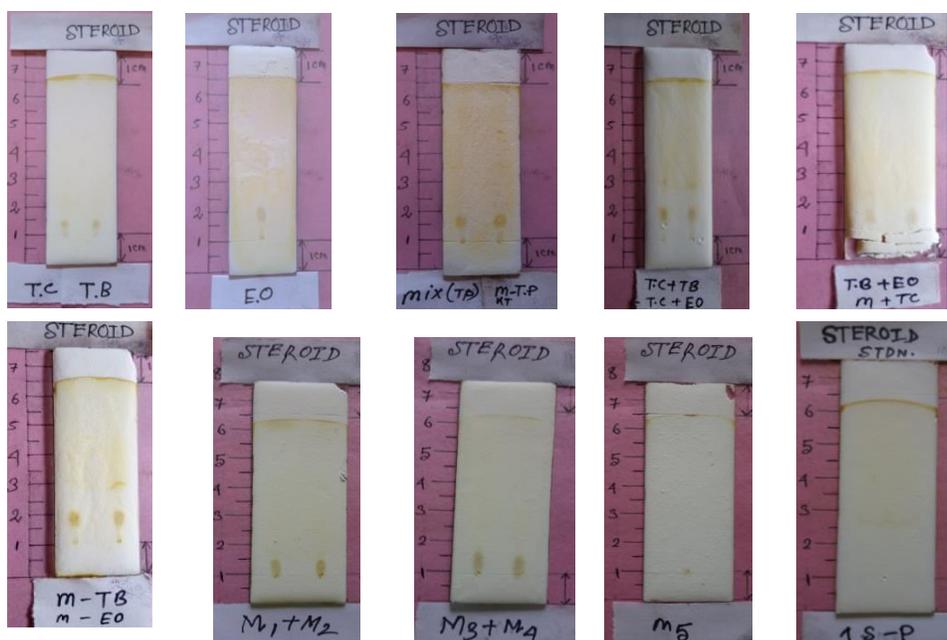


Figure 3. Steroid TLC profiles of various combinations and market samples.

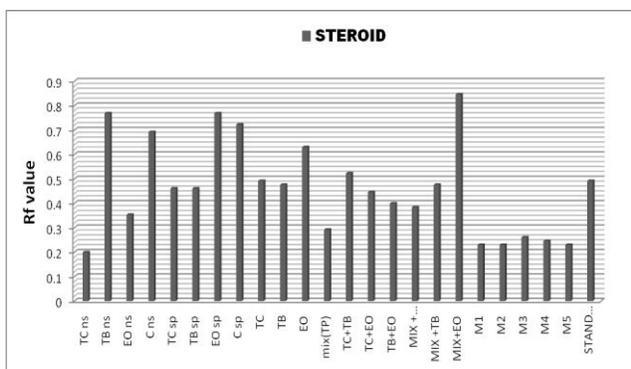


Figure 4. Graphical presentation of TLC for Steroid.

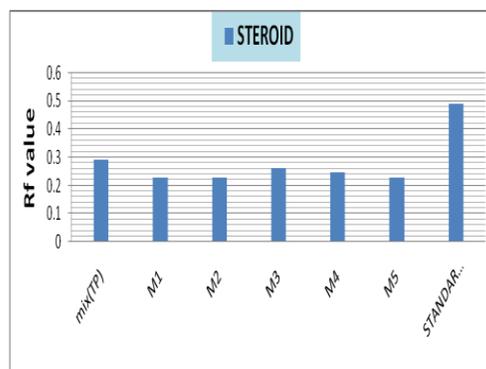


Figure 5 Steroid TLC of customized and Market samples

STERIOD TLC ANALYSIS: From the Steroid TLC profiles of only peels of three fruits and their mixture, it was observed that steroid was present in all the four samples. The maximum rf value of (0.769) was observed in TB and which matched with similar value in the mixture. This indicated that steroid present in TB represented of the mixture as compared to other two peels.

The steroid profiles of the seeds and the mixture indicated that EO had two spots (Rf - 0.230 and 0.769) whereas that of TB and TC showed only one spot (rf-0.461). The mixture showed two spots (Rf - 0.230 and 0.723). These results indicate that the steroid present in EO was more expressive as compared to those of TB and TC (Rf - 0.461) which were totally masked by EO's. The same value of 0.461 for both TB and TC indicate similar type of steroids present in them and this also could be a reason for the lowering of the rf value of second spot value(0.723 as compared to that of 0.769).

Steroid profiles of whole dried fruits indicated that rf values for steroids in the fruits were in the order of EO (0.630), TC (0.492) and TB (0.476) respectively. The results indicate that the steroid present in EO are different than those in TC and TB which could be similar due to their near similar Rf values.

The Rf value of steroid was lower in the mixture when compared to those of individual constituents, Csp and Cns. This lowering of Rf value in the mixture could be the variation in concentrations of steroid in the components or due to some masking effect among them.

To understand this anomaly, TLC was performed on mixtures of only two ingredients such as TC+TB, TC+EO and TB+EO. It was observed that TC+TB

showed an Rf value at 0.523 which was higher than their individual Rf values (0.492 and 0.476), respectively. The Rf value for TC+EO was lower than their individual values. This variation could be due to the masking effect of TC and TB over EO.

To test whether there was any difference in the rf values if each ingredient was double in the mixture at a time, such as MIX+TC, MIX+TB & MIX +EO, it was observed that the rf values of mixtures were less compared to the individual components except mix+EO, which indicated more rf values ((0.630 to 0.846). These results could indicate that the increase in the rf value when EO was doubled in the mixture could be due to higher presence on steroid in EO.

Further, the customized mixtures of *Triphala*, without seeds (Cns) and with seeds (Csp) and whole fruits (Mix-TP) were compared with various market samples of *Triphala*. This was done with an idea to know whether the markets samples were prepared without seeds or with seeds. It is important to mention here that *Triphala* mixture is supposed to be without seeds of the three ingredients. It was quite interesting to see that the rf values of Cns and Csp were nearer to each other (0.692 and 0.723) whereas the rf values of (Mix-TP) and those of market samples (M1-M7) ranged nearer to (Mix-TP, i.e. mixture of whole fruits). The results clearly indicate that all the market samples were prepared with whole fruits and not with the peels alone. The steroid profile indicate that the seeds alone showed higher rf value when compared to the peels (i.e. 0.723 and 0.692) whereas the whole fruit mixture showed very low Rf values (Around 0.23 to 0.29) including the market samples. The reason for taking only peels for the preparation of *Triphala* by Ayurveda has to be studied.

Terpenoid TLC Profile Study. Figure 6, 7, 8 and 9 indicate the TLC profiles of Terpenoids of samples.

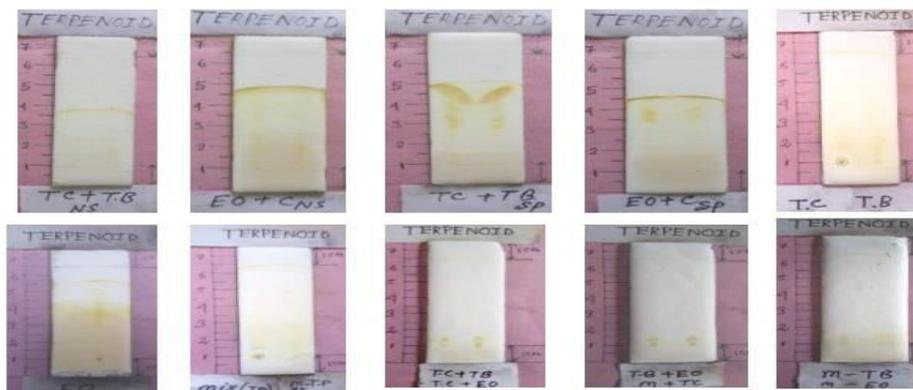


Figure 6. Terpenoid TLC profile of *T. chebula*, *T. bellerica* and *E. officinalis* rinds and seeds.

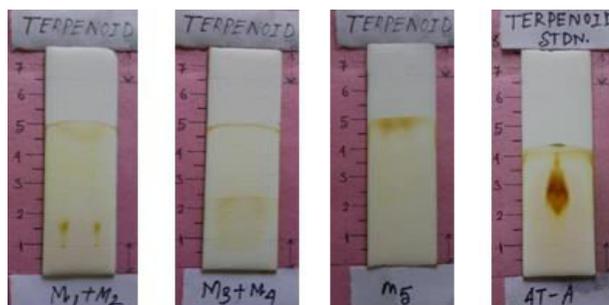


Figure 7. TLC of Terpenoid of Market samples

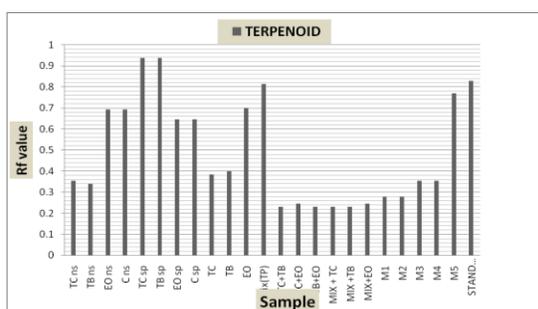


Figure 8. Graphical representation of TLC of Terpenoid of various samples

For the analysis of Terpenoids the materials selected were only rinds, only seeds and whole fruits. TLC profile for only rinds among all three ingredients, EOns showed more Rf value (0.692) than the other two TCns (0.353) and TBns (0.338). It was also observed that EO indicated three spots while the mixture of the three also indicated one spot matching with EO (rf-0.692) i.e. EO expressed more in the mixture and masked the other two although both were present clearly as indicating by their rf values.

When the same process was done for seeds alone, the rf values of TCsp and TBsp matched with each other (0.507 and 0.939) as compared to EOsp (0.646) indicating that the terpenoids present in TCsp and TBsp were different from that of EOsp. But the rf value of the mixture of three seeds was similar to that of EOsp (rf-0.646) indicating that EO terpenoid has masking effect on other two.

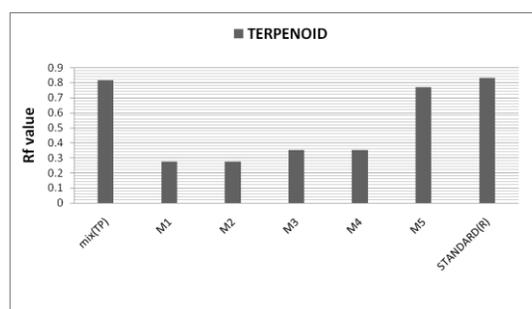


Figure 9. TLC profiles of Market samples compared to Standard

The TLC profiles of whole fruits and their mixture showed that TC and TB was lower than EO (0.70) whereas the mixture showed higher rf value (0.815) i.e. terpenoid molecule present in peel and seeds are different and they act differently in the mixture, masking effect of EO.

Likewise, the TLC profiles of TC+TB, TC+EO, TB+EO, MIX+TC, MIX+TB, MIX+EO and several market samples were analyzed. The rf values were found to be lower than that of individual as well as the customized sample (Csp, Cns, mix-TP) and also from the standard. Only in one Market sample (M5) the rf was nearer to that of Mix+TP.

With the above terpenoid profiles it is clear that whole fruit mixture to make triphala has different rf value when compared to the standard and than that of customized sample.

Flavonoid TLC Profile Study. Figure 10, 11, 12, 13, 14 and 15 indicate the TLC profiles of Flavonoids of samples.

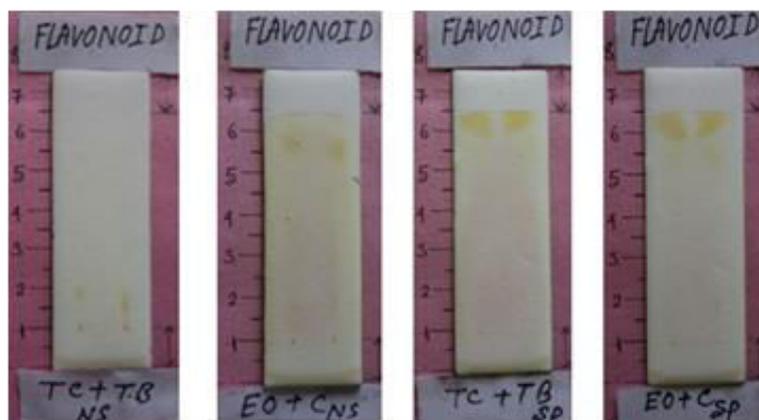


Figure 10. Flavonoid TLC profile of *T. chebula*, *T. bellerica* and *E. officinalis* rinds and seeds.



Figure 11. Flavonoids TLC profile of *T. chebula*, *T. bellerica* and *E. officinalis* fruits and mixture of three fruits.

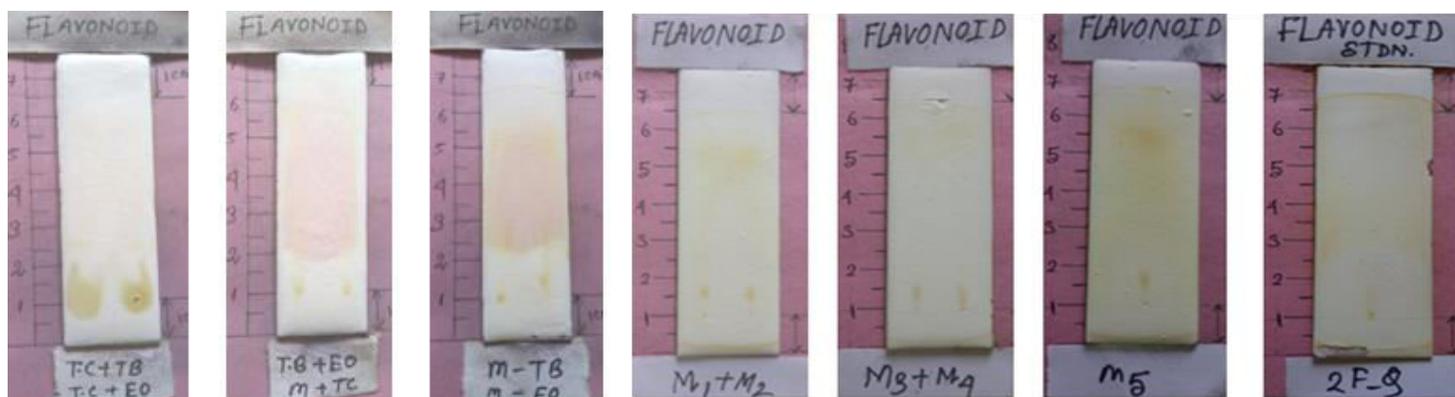


Figure 12. TLC of Flavonoid after doubling one constituent to TC and market samples

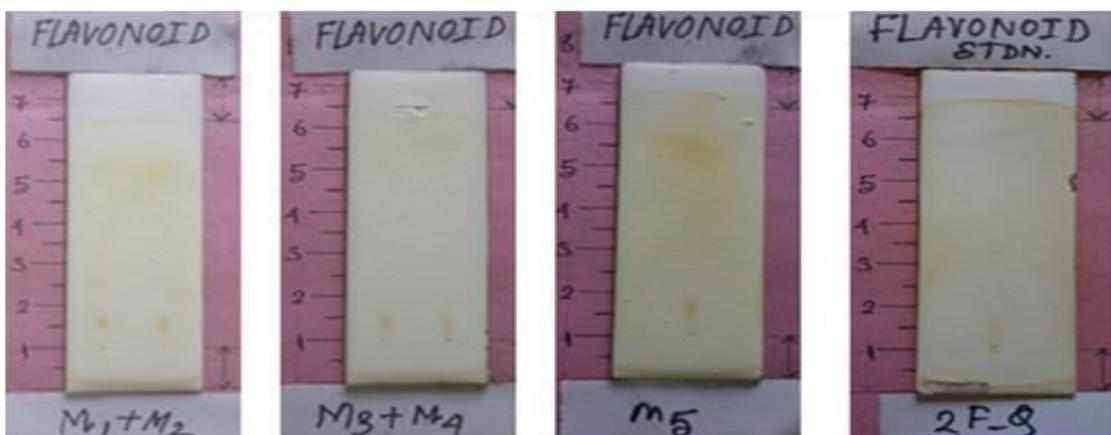


Figure 13. Comparison of TLC of Flavonoids of market samples with Standard

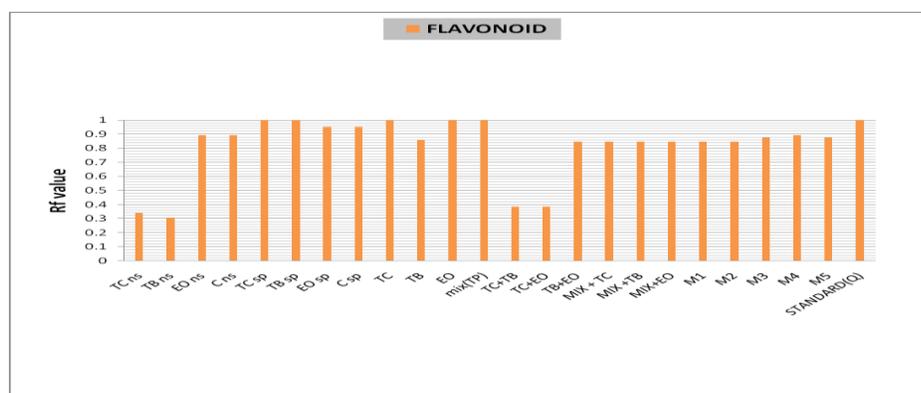


Figure 14. Graphical representation of Flavonoid TLC profile of TC.

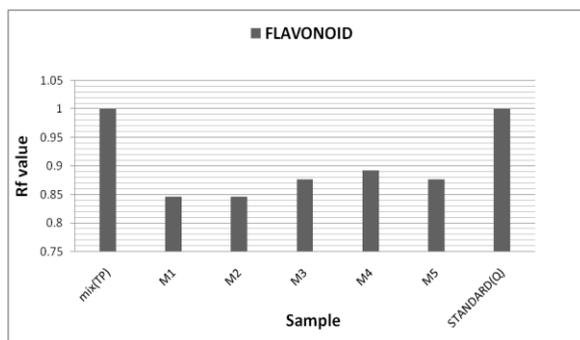


Figure 15. Graphical representation of Flavonoid rf values of customized and Market TC.

FLAVONOIDS

The TLC rf values of Flavonoids were 0.892, 0.963, 0.892 and 0.953 for Cns, Csp, Eons and EOsp respectively. The similarity was also found when the whole fruits and were carried with TLC (EO=1, MIX-TP= 1) and also for the mixture of TB+EO=0.846 and the masking effect observed for TC over TB & EO and EO over the TB and for mixture while respective ingredients present double in quantity. The rf values of Customized triphala, standard terpenoid and market sample M4 was same. The other market samples ranged from 0.846(M1, M2) and 0.876 (M3, M5)

Alkaloid TLC Profile Study. Figure 16, 17, 18, 19, 20 and 21 indicate the TLC profiles of alkaloid of samples.

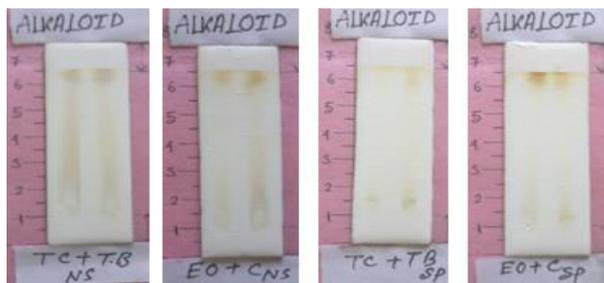


Figure. 16. Alkaloid TLC profile of rinds and customized sample of *Triphala*



Figure. 17 Alkaloid TLC profiles of fruits.

Figure.18 Alkaloid TLC profile by doubling each ingredient



Figure. 19 Alkaloid TLC profiles of Market samples and standard

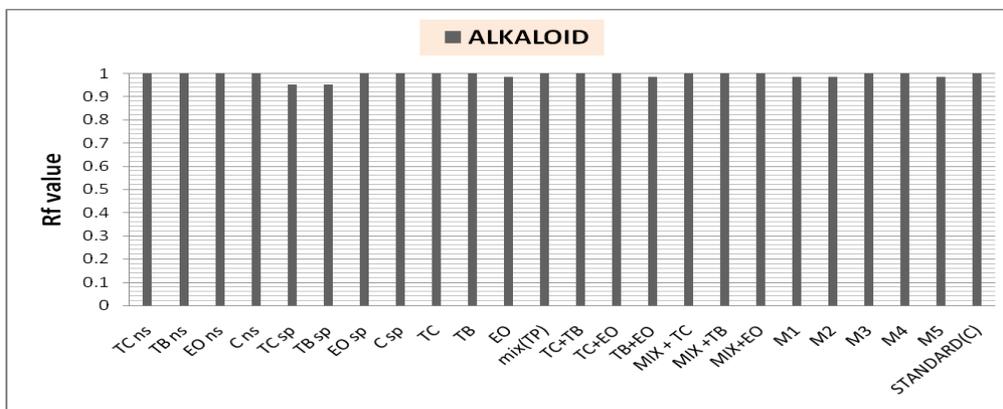


Figure. 20 Graphical representation of Alkaloid TLC profile of TC.

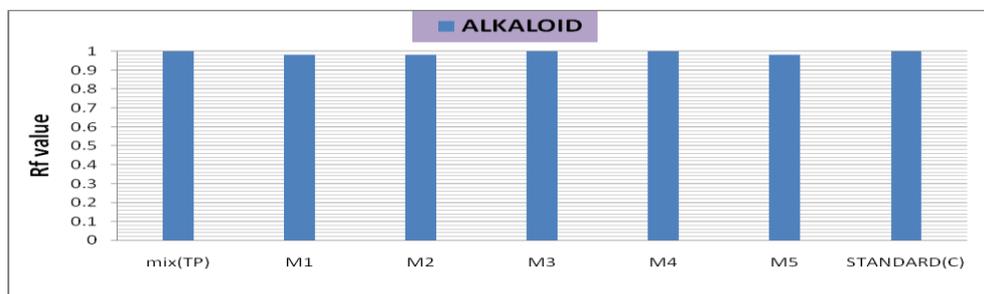


Figure 21. Alkaloid TLC profiles of market samples and standard.

ALKALOID

The TLC profiles of alkaloids for only peel, only seed, mixture of all peels, mixture of all seeds, whole fruits and by mixing of two separately, mixture of all fruits had same Rf value (1) which was nearer to the Rf value of standard (0.984) indicated that alkaloids molecules present in all were more or less similar molecules and with similar polarity.

It was interesting to observe that the rf values were exactly same for Cns, Csp, MIX-TP (customized) and market sample M1, M2 and M5 (0.984). Cns, Csp, Mix-TP, standard, M3 and M4 had similar rf values (1.000) indicating that the market sample were made of whole fruits. Among all phyto-chemicals namely steroid, terpenoid, flavonoid and alkaloid the Rf value which were obtained for all ingredients by different experimental process was maximum only for alkaloids.

The TLC profiles of standards are shown in Figure 22 and the comparative TLC profiles of ingredients with and without seeds as compared to the respective standards is shown in Figure 23.

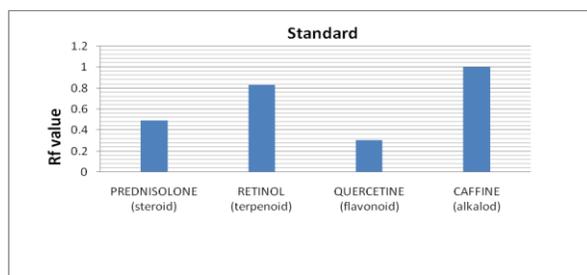


Figure 22. TLC profiles of standards.

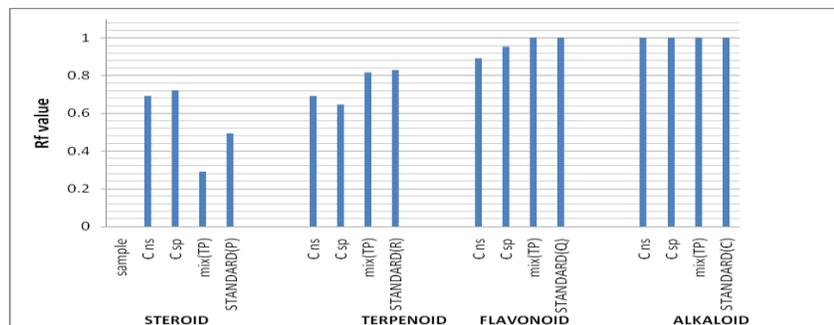


Figure 23. Graphical representation of TLC rf values ingredients and TC as compared to Standards of all the secondary metabolites.

CONCLUSION:

The TLC profiles of steroids, terpenoids, flavonoids and alkaloids indicated different behaviours for only rinds, only seeds, their respective mixtures, whole fruits separately and their mixtures.

It was observed that alkaloids were almost similar in all the samples indicating their predominance in *Triphala* ingredients as well as those of mixtures. *Embelica officinalis* was having more dominating and masking effect over the other two constituents for all the four metabolites studied. It was also clear that most of the market samples indicated the presence of whole fruits as their raw materials instead of the rinds alone, which needs to be addressed. From the study it is clear that this methodology can be used to estimate the presence of the constituents in each sample and suggest this easy method for quality analysis of the samples before they come to market. By doing so standard medicine will come to market for public which is healthy and without any negative health impact.

COMPETING INTERESTS

This is to inform that no conflict of interest exists among the author.

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