Qualitative and quantitative analysis of phyto-chemicals with estimation of targeted metabolite; piperine in *Shwaskuthara rasa*

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Abstract: *Shwaskuthara Rasa* is used for Bronchial asthma**,** allergies, is used for the cure of cough, laryngitis, tuberculosis, unconsciousness, mental disorders, comma, chest burn, and heart diseases. It is a herbo-metallic formulation having organic and inorganic components. Every bio-active compounds have their respective role in pharmacology of the drug. Present study is planned to detect and quantified phyto-chemicals present in the drug. Furthermore, a targeted metabolite named piperine was estimated through sophisticated instruments.

1. Introduction:

Traditional medicine, according to the World Health Organization, encompasses the accumulated knowledge, skills, and practices rooted in the theories, beliefs, and experiences specific to various cultures, regardless of their explainability, utilized in maintaining health as well as in preventing, diagnosing, improving, or treating physical and mental ailments.[1,1] The systems of traditional medicine are backed by extensive literature and documented theoretical frameworks and practical skills. The wisdoms are transmitted through oral traditions from one generation to the next. In recent time, many people depend on their indigenous traditional medicine for their primary health care requirements.[2] Ayurveda is a distinct and self-sustaining medical system that has endured throughout history. While the exact origins of Ayurveda remain obscure in ancient times, its defining principles seem to have developed in India between 2500 and 500 BC. It is recognized as a holistic therapeutics which ensure the well being of humanity through the natural resources. The drug compendium of Ayurveda comprises thousands of formulations made up of herbs, metals, minerals and animal products. The formulations are categorized into herbal, herbo-mineral and metallo-mineral categories. Herbo-metallic and metallo-mineral drugs are considered as superior to the herbal drugs as they exhibit greater and quick absorption, distribution and assimilation. They are mentioned to have multi -dimensional pharmacological actions depending upon the character of the vehicles. Herbo-metallic drugs are preferred and prescribed extensively than metallo-mineral drugs in recent times for its multidimensional use and relatively low risk as herbal components are established to neutralize the untoward effect generated by metalloid.[3] “Shwaskuthara Rasa” as

the name itself indicates that it is the mercurial preparation, which acts as an axe to *Shwasa Roga* and helps in eliminating the disease *Shwasa* (Asthma and allergic conditions) from its root. It is first explained in *Rasendra Sara Samgraha* in 15th century. Apart from treating asthma and allergies, is used for the cure of cough, laryngitis, tuberculosis, unconsciousness, mental disorders, comma, chest burn, and heart diseases. This drug comprises processed mercuric sulfide, processed arsenic disulfide, processed borax as inorganic ingredients and purified aconite, dry ginger, long pepper and black pepper as organic ingredients. As per Ayurveda Formulary of India, it consists of 56.25% of black pepper which is mentioned to be incorporated in the drug by adding and triturating one by one. This process is very unique regarding the pharmaceutical processing and believed to provide specific pharmaco-kinetics and dynamics. Additionally, it is an example of arithmetic dilution and activation of bio-active compounds. Piperine is present in black pepper (Piper nigrum), white pepper, and long pepper (Piper longum), which are part of the Piperaceae family. It exhibits a variety of biological effects, including anti-inflammatory, anticancer, antiviral, anti-larvicidal, pesticide, anti-Alzheimer's, antidepressant properties, and notably, it is recognized as a bioavailability enhancer. The quantity of pperine is very much important to generate evidence regarding the pharamco-kinetics and pharamaco-dynamics of this drug.[4] Present study is planned to determine its quantity through sophistical instrument and review other micro-matrix components along with pharmacological actions of *Shwaskuthara rasa*.

2. Material and methods:

2.1 Preparation of test drugs:

SKR was prepared as per the classical method mentioned in AFI[56]. All the ingredients (Table no. 1) were purchased from the Kharibauli market, New Delhi and authenticated by the experts of *Drayaguna* (Ayurvedic pharmacogonosy & pharmacology) before use.

Table No. 1: Ingredients of SKR and their part used

Sl. No.	Content	Part use	Chemical composition/Botanical name	Ratio
1.	*Shuddha Parada* (Processed Mercury)	Inorganic compound	Mercury (Hg)	1 part
2.	*Shuddha Gandhak* (Processed Sulfur)	Inorganic compound	Sulfur (S)	1 part
3.	*Shuddha Vatsanabh* (Processed Aconitum)	Root Tuber	*Aconitum chasmanthum* Staph. ex Holmes.	1 part
4.	*Shuddha Tankan* (Processed Borax)	Dehydrated	Sodium borate ($Na_2B_4O_7$, 10 H_2O)	1 part
5.	*Shuddha Manashila* (Processed Realgar)	Inorganic compound	Arsenic di sulfide (As_2S_2)	1 part
6.	*Shunthi* (Dried Ginger)	Rhizome powder	*Zingiber officinale* Roscoe	1 part

7.	*Pippali* (Long pepper)	Fruit powder	*Piper longum* L.	1 part
8.	*Marich* (Black pepper)	Fruit & Fruit powder	*Piper nigrum* L.	9 part

2.2 Qualitative screening of Phyto-chemicals: Organic solvents of different polarities were used to extract phyto-chemicals present in the formulation. Methanol, ethyl acetate and petroleum ether extracts of VG have been tested for the presence of Alkaloid[7], flavonoid[8], saponins[9], steroid[10] and glycosides[11] by following standard method and guidelines.

2.3 Quantitative estimation of phyto-chemicals: Total phenolic content (TPC) of Methanol extract was quantified as per Folin ciocalteu method through UV-Vis spectroscopy[12]. The sample was analyzed in triplicate at 517 nm wavelength while the standard curve was created by using Gallic acid at various concentrations ranging from 5-100 **µg/ml** in methanol.

2.4 FTIR (Fourier Transform Infrared Spectroscopy): The test drug was analyzed for presence of piperine and other functional groups by FTIR **(**Bruker, 3000 Hyperion Microscope with Vertex 80 FTIR System, Germany) and the spectra were taken in the region of 4000-400 cm^{-1}[13]. The spectra obtained from the pierine matched with the spectra produced by the test drug.

2.5 Estimation of targeted metabolite: The test drug consists of large amount of piperine. which was quantified through High performance thin layer chromatography (HPTLC) with reference standard in the present study. Test samples were prepared at a concentration of 10mg/ml in methanol followed by sonication for 20 minutes, while the piperine (98% pure Sigma-Aldrich) reference standard was prepared in same solvent at a concentration of 1mg/ml. Then sample in triplicate (6, 8 and 10 **µl)** and standard in 2-9 **µl** were applied for generation of calibration curve on the TLC plates silica gel 60 F 254 (stationary phase) by automatic sample Applicator (CAMAG Linomat, Spray gas-Inert gas, Sample solvent type-Methanol, Dosage speed-150 nl/s, Syringe size- 100 ml, Band width- 8.00 mm). Mixture of Toluene: Ethyl acetate: Acitic acid (6:4:0.1), was used as mobile phase. Scanning was performed at (λ= 254nm, 366 nm) and quantification (λ= 330 nm) were done by CAMAG TLC scanner having specifications of (Scanner_230698(2.01.02), Slit dimensions- 6.00×3.00 mm, micro, Scanning speed- 20nm/s, Wavelenth-254 nm, Lamp-D2, Measurement type- Remission, Measurement mode- Absorption).

3. Result:

3.1 **Qualitative screening of phyto-chemicals**: Steroid was not found in any of the tested extracts. Ethanol and ethyl acetate extracts have shown the presence of alkaloid, flavonoid, glycoside, saponnin and tanins. Glycosides is found to be rich in aqueous solvent.

Table 2: Result obtained in functional group analysis: (+ Present, - Absent)

Test	Ethyl Acetate	Ethanol	Aqueous
Alkaloids	+	++	+
Steroids	-	-	-
Flavonoids	+	++	+
Glycosides	+	+	++
Saponin	+	++	+
Tanins and phenolic compound	+	+	-
Proteins	-	-	-

3.2. Total phenolic content (TPC): TPC was calculated as 6.54± 0.82 µg/g/GA while standard curve was developed with different concentration of Gallic acid (R^2= 0.9962).

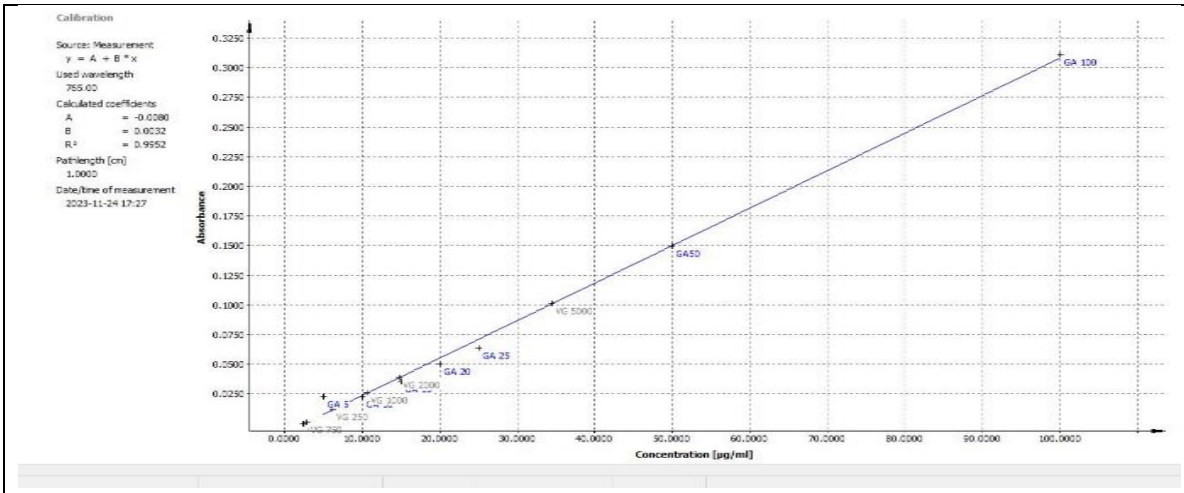

Fig no 1: Standard curve gallic acid for TPC estimation

3.4 FTIR: Scanning in IR light (4000-400 cm^{-1} wavelength) detected 8 peaks in the test drug where 4 peaks were detected in piperine standard.

Table 3: Obtained peaks of SKR and Piperine

Standard peak zone	Obtained peaks in SKR	Obtained peaks in Piperine	Bond	Functional group	Types of bond
3540-2700 **3400-3100**	3363.36	3338.25	O-H N-H	Alcohol Ammonia	Strong, broad and stretching vibration Medium, stretching vibration

1500-1470	1491.47		C-H	Alkyl	Medium deformation vibration(Asym)
1465-1440	1448.08	1446.24	C-H	Alkyl	Medium deformation vibration(Sym)
1370-1390	1383.87		C-H wag	Alkylhalide	Strong-medium stretching vibration
1365-1295	1347.96`	1253.63	C-H wag	Alkyl halide	Strong streaching vibration
1150-1130	1131.77	1019.08	C-C	Alkene	Medium rocking vibration
1085-1030	1079.51		C-O	Alcohol	Strong stretching vibration
1000-960	998.92		C-C	Alkene	Weak skeleton vibration

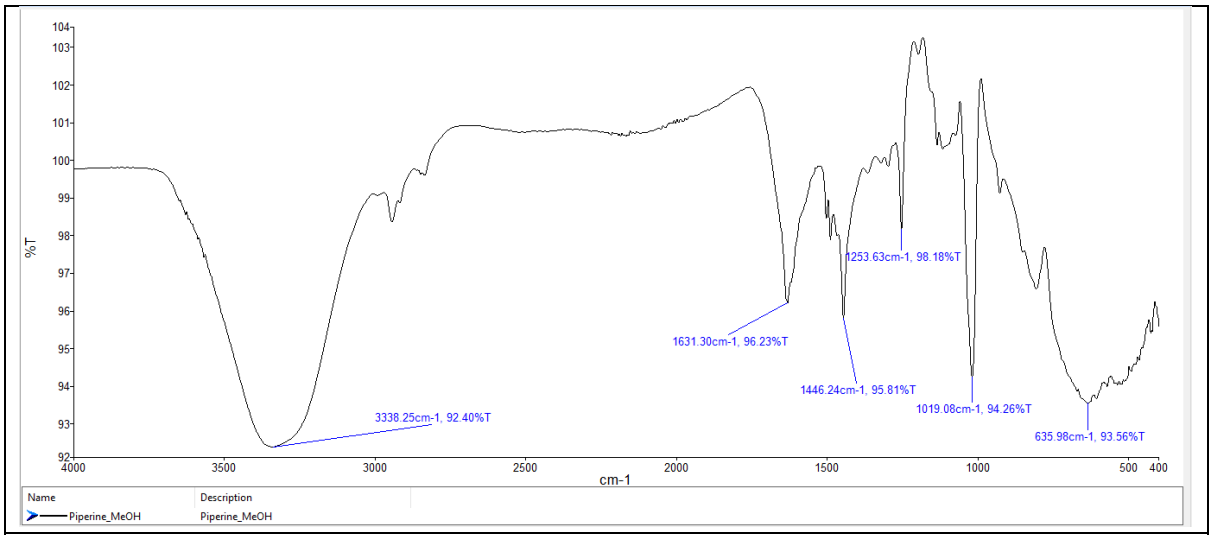

Fig no 2: FTIR spectra denoting different functional group in Piprine

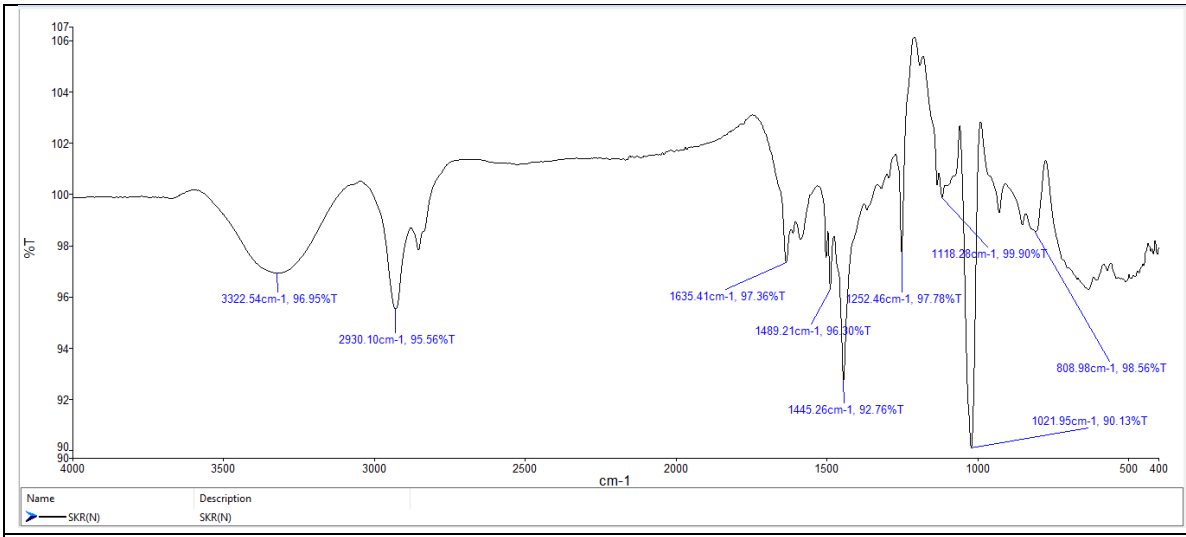

Fig no 3: FTIR spectra denoting different functional group in SKR

3.3 Detection and quantification of piperine:

Separation of piperine and SKR was well observed in 254 & 366 nm respectively. Initially scanning was performed at 254 & 366 nm and piperine was identified at R_f value of 0.47±0.047 (254 nm). After assigning the reference substance spectra was taken between 254 to 450 nm. The observed λ max was noted as 330 nm and on quantitation, piperine of 9.59 µg/mg was quantified in the test drug with CV of 1.76%.

Table 4: Number of spot, R_f value which were obtained at 254 nm wavelength

Position of band	Name of sample	No of spots	R_f value
1	MeOH of SKR	12	0.01, 0.05, 0.25, 0.29, 0.34, 0.39, 0.47, 0.53, 0.62, 0.70, 0.80, 1.01
2	MeOH of SKR	11	0.01, 0.24, 0.29, 0.33, 0.38, 0.47, 0.53, 0.61, 0.69, 0.78, 1.01
3	MeOH of *Maricha*	10	0.01, 0.25, 0.29, 0.33, 0.39, 0.46, 0.52, 0.62, 0.69, 0.84, 1.01
4	Pipirine (2µg/ml)	5	0.29, 0.46, 0.51, 0.84, 1.00
5	Piperine (3µg/ml)	4	0.28, 0.45, 0.83, 1.00
6	Piperine (4µg/ml)	5	0.28, 0.45, 0.71, 0.83, 1.00
7	MeOH of SKR	12	0.00, 0.11, 0.24, 0.28, 0.32, 0.38, 0.46, 0.52, 0.60, 0.68, 0.77, 1.0
8	MeOH of *Maricha*	12	0.00, 0.14, 0.24, 0.28, 0.32, 0.37, 0.45, 0.51, 0.61, 0.68, 0.83, 1.00
9	MeOH of *Maricha*	11	0.00, 0.24, 0.28, 0.32, 0.37, 0.45, 0.51, 0.61, 0.68, 0.83, 1.00

10	Piperine (5µg/ml)	6	0.01, 0.27, 0.45, 0.77, 0.82, 1.00
11	Piperine (6µg/ml)	5	0.00, 0.28, 0.45, 0.83, 1.00
12	Piperine (7µg/ml)	5	0.00, 0.27, 0.45, 0.83, 1.00
13	Piperine (8µg/ml)	5	0.00, 0.27, 0.45, 0.82, 1.00
14	Piperine (9µg/ml)	6	0.01, 0.27, 0.45,0.64, 0.82, 1.00

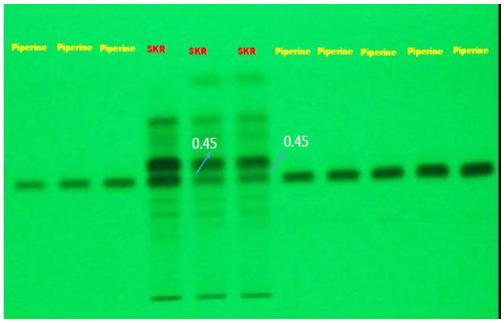

Fig 4: Piperine at 254 nm

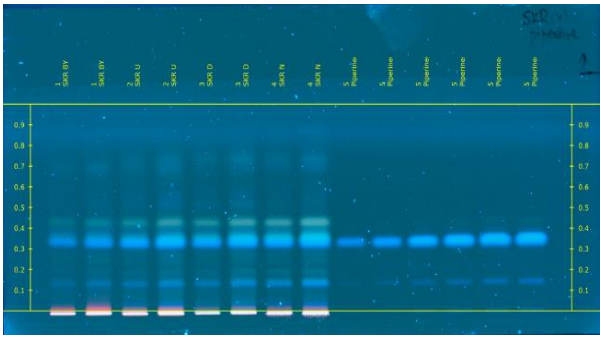

Fig 5: Piperine at 366 nm

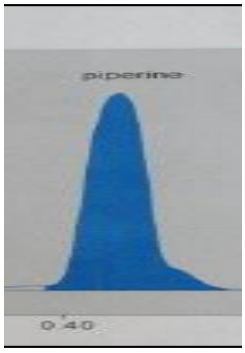

Fig 6: Curve with AUC of Piperine

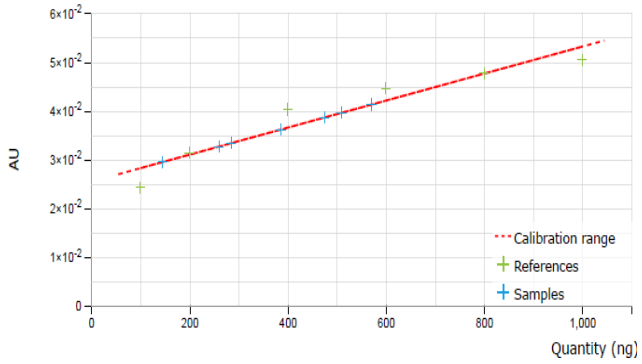

Fig 7: Linerity of different conc. of piperine

4. Discussion: Phytochemicals of different groups viz. alkaloid, flavonoid, glycoside and saponin were detected in different organic media. Each one has their own pharmacological actions through different pathway to mitigate various patho-physiology. These substances are referred to as secondary plant metabolites and offer health advantages for humans. It is believed that they function as synergistic agents, enabling the body to utilize nutrients more effectively. Some of the positive roles of phytochemicals include low toxicity, affordability, easy accessibility, and their biological characteristics, such as antioxidant functions, antimicrobial properties, regulation of detoxification enzymes, enhancement of the immune system,

reduction of platelet clumping, and regulation of hormone metabolism along with anticancer properties.[14] The presence of various phyto-chemicals in SKR indicate its multi-dimensional pharmaco-dynamics. Total phenolic content is the cumulative of simple phenolics (resorcinol and phloroglucinol), phenolic acids, aldehydes, coumarins, flavonoids, chalcones, aurones, benzophenones, xanthones, stilbenes, benzoquinones, naphtha-quinones, anthraquinones, betacyanins, lignans, and polyphenols[15]. TPC of SKR is found to be 6.54± 0.482 µg/g/GA. They mainly impart anti-oxidant property[16]. Besides that, these are known to have anti-inflammatory, anti-microbial, anti-viral, anti-fungal activities which can be key factors to mitigate different pulmonary pathologies. [17] A recent study showed polyphenols suppressed inducible nitric oxide synthase expression, and prevented oxidative and nitroxidative lung injury. Furthermore, it down-regulated cyclo-oxygenase-2, extracellular signal-regulated kinase phosphorylated expression which having impact on pulmonary cellular damage.[18] FTIR revealed SKR having organic substances of different functional groups viz. Alcohol (O-H), Alkyl (C-H), Alkene (C-C), Alkylhalide (C-H wag). The detected O-H groups indicate the presence of phenols in the test drug[19]. Alcohol (O-H), Alkyl (C-H), Alkene (C-C), aliphatic alkene (C=C) groups are found to be common in piperine standard and SKR. After detection the piperine in the test drug, HPTLC was applied to determine the quantity. The sophisticated chromatography method quantified 9.59 µg/mg of piperine in SKR. Piperine is a amide alkaloid, isomer of chavicine displays various biological actions. Recent studies indicate that piperine exhibits a broad range of biological activities, including the stimulation of pancreatic digestive enzymes, the inhibition of oxidation processes initiated by free radicals, and the enhancement of the bioavailability of various therapeutic medications. Furthermore, piperine has shown to possess anti-inflammatory properties in several models of autoimmune inflammatory diseases, such as inflammatory bowel disease, arthritis, type 1 diabetes, and cancer. Piperine activates the transient receptor potential vanilloid type 1 receptor and influences GABAA receptors. At comparable concentrations, piperine inhibits both monoamine oxidases (MAOs), specifically MAO-A and MAO-B. Similar to other natural compounds with methylenedioxyphenyl substituents, piperine impacts cytochrome (CYP) isoforms, inhibiting the CYP3A isoform while enhancing the expression of CYP1A and CYP2B in the liver. Additionally, it exhibits a biphasic effect on the activity of P-glycoprotein. Piperine has also been noted to modulate cell signaling pathways, including the NF-kB pathway. It also alleviates pulmonary inflammation by modulating the SIRT1-mediated inflammatory cascade, inhibits epithelial–mesenchymal transition, and activates Nrf2 signaling. Another study suggest Piperine effectively alleviates asthma by decreasing levels of Th2 cytokines (such as interleukin-4 and interleukin-5), minimizing eosinophil infiltration, and significantly lowering the expression of thymus and activation regulated chemokine, eotaxin-2, and interleukin-13 mRNA in lung tissue. It also reduces the levels of interleukin-4, interleukin-5, and eotaxin in bronchoalveolar lavage fluid, as well as the production of histamine and ovalbumin-specific immunoglobulin E in serum.[20] piperine inhibited mitochondrial tricarboxylic acid cycle, phase-I, and glutathione metabolizing enzymes in benzo(a)pyrene-induced experimental lung carcinogenesis in Swiss albino mice. Piperine has the capability to alter the metabolism of supplements and medications. This usually enhances the bioavailability of compounds and prevents glucuronidation, a liver process that attaches a glucuronide molecule to drugs, indicating that they should be excreted through urine. Piperine

has the ability to enhance the bioavailability of a variety of drugs that have different structures and therapeutic roles. The bioavailability-enhancing effects of piperine may be linked to improved absorption, which could stem from changes in the dynamics of membrane lipids and alterations in the conformation of intestinal enzymes.[21] Additionally, piperine increases the activity of leucine amino peptidase and glycyl-glycine dipeptidase, as a result of changes in enzyme kinetics. This indicates that piperine might influence membrane dynamics due to its nonpolar characteristics by interacting with nearby lipids and the hydrophobic areas of proteins, potentially altering enzyme conformations.[22] From the above discussion it can be inferred that, SKR having piperine as bio-active compound has immense potential to mitigate various pathological conditions specially the pulmonary pathology. Additionally, the pharmaco-kinetics of the SKR can be intensively influenced by the presence of piperine as it have crucial effect on the bio-enzymes.

Conclusion: Shwaskuthar Rasa is a herbo-metallic formulation having various organic bioactive compounds. Piperine is one of them which is found to be present in SKR at 9.59 micogram/mg. The organic bio-ative compounds assure the multi-dimensional pharmacology of SKR. The pharmaco-dynamics of SKR can be explained by the presence of piperine. Furthermore, it can be a lead to evaluate the pharmao-kineticof the SKR.

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