

Research Article

GLOBAL METABOLITE PROFILING AND NETWORK PHARMACOLOGY OF TRIPHALA IDENTIFIES NEUROMODULATORY RECEPTOR PROTEINS AS POTENTIAL TARGETS

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Abstract: Triphala is an herbal formulation widely used in Ayurvedic medicine as a rejuvenation and revitalization therapy. Its constituents include a mixture of dried extracts from *Phyllanthus emblica*, *Terminalia chebula* and *Terminalia bellirica*. This formulation has been extensively investigated for its anti-cancer, anti-caries, anti-microbial, immunomodulatory and hypolipidemic activities. Currently, the activity of Triphala is attributed to the composition of its individual constituents. However, its composition has not been comprehensively characterized. In this study, we employed a mass spectrometry-based strategy to globally profile the constituent metabolites in Triphala. Using methanolic and aqueous extraction methods, we identified 1,897 metabolites in Triphala. We identified previously known Triphala metabolites such as gallic acid derivatives, chebulinic acid, syringic acid, ascorbic acid derivatives and epicatechin. We also identified several metabolites that were not previously reported from Triphala extract including metabolites from isoquinoline alkaloids, flavonoid, stilbenoid, diarylheptanoid and gingerol and indole alkaloid classes. Pathway analysis revealed enrichment of thiamine metabolism, tyrosine metabolism, lysine biosynthesis and isoquinoline alkaloid biosynthesis pathways. Furthermore, prediction of binding targets of Triphala metabolites using a network pharmacology approach resulted in the identification of 387 potential protein targets in human, mammalian and microbial species suggesting potential anti-microbial, anti-cancer, and nootropic activities. Protein targets of Triphala include members of neuromodulatory classes including opioid receptor, 5-Hydroxytryptamine receptor, alpha adrenergic receptor, glutamate receptor and kinases including mTOR, p38, Aurora kinase B AXL, VEGFR, PI3K, PKC among others. The metabolite profiling of Triphala is a proof-of-principle study that will serve to benchmark, standardize and characterize other traditional formulations.

Keywords: Traditional medicine; metabolic fingerprinting; drug benchmarking.

Note - Coloured Figures and Supplementary Information are available on Journal Website in "Archives" Section

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Introduction

The use of herbal formulations as remedies and treatments for various illnesses has been gaining global attention. Among these, the Ayurvedic system of traditional medicine is being practiced from time immemorial for the treatment and/or management of several ailments. Several studies have focused on elucidating the mechanism of action of Ayurvedic formulations and the identification of known active principles. However, the effects, efficacy and constituents of formulations used in Ayurveda are not well understood.

The *Rasayan chikitsa* system of medicine in Ayurveda uses polyherbal preparations, which are used in rejuvenation and revitalization therapy. Triphala is one such popular Ayurvedic formulation used to treat various disorders. The formulation consists of dried extracts of *Phyllanthus emblica* (*Emblica officinalis*), *Terminalia bellerica* and *Terminalia chebula* (Chandran *et al.*, 2015). Triphala and/or its individual plant constituents have been reported to possess antibacterial, antimalarial, antifungal, antiallergic, and antiviral activities (Singh *et al.*, 2008; Srikumar *et al.*, 2007). Owing to these properties, it is used in the treatment of cardiovascular disorders, ophthalmic problems, liver dysfunction, inflammation and complications of the large intestine (Singh *et al.*, 2015; Yoon *et al.*, 2012). Triphala has also been reported to be effective in treating cough, asthma, fever, chronic ulcers, leucorrhoea and pyorrhea. Triphala is a cardiotonic and has a potential to improve blood circulation, reducing myocardial necrosis and serum cholesterol levels, and strengthening the capillaries. Triphala has been reported to possess anti-aging properties and to improve the mental faculties, regulate the adrenergic function and therefore, therefore it enables the body to recover from stress. Several studies performed in the past decade suggest the active compounds of Triphala show antioxidant, antidiabetic (Sabu *et al.*, 2002), anticancer (Lu *et al.*, 2012), antiproliferative (Sivasankar *et al.*, 2015), antimutagenic (Kaur *et al.*, 2002), radioprotective (Takauji *et al.*, 2016), and chemopreventive activities (Deep *et al.*, 2005). Several groups have carried out randomized controlled trials to assess the effect of Triphala on dental health. Aqueous preparation of Triphala mouth wash has been compared with chlorohexidine formulated commercially available mouthwashes. These studies have proven the benefits of Triphala comparable with that of the chlorohexidine containing mouth wash, in terms of

prevention of plaque buildup and thereby the management and prevention of periodontal problems (Bajaj *et al.*, 2011; Bhattacharjee *et al.*, 2015; Naiktar *et al.*, 2014; Narayan *et al.*, 2012). It has also been established to confer enteroprotective (Nariya *et al.*, 2009) and gastroprotective effects (Nariya *et al.*, 2011).

Given the varied role of Triphala in treatment and management of several disorders, it is vital to identify the components that can be ascribed to its activity. Furthermore, the formulations are generally short lived and batch-to-batch variation exists. Knowledge of the constituents of a formulation and its amounts are necessary to minimize variation and effect caused thereof. The activity of *Triphala* is attributed to the composition of its individual constituents i.e. the myrobalans. *Triphala* being a compounded formulation, a single biochemical component cannot be ascribed to its activity. Some notable phytochemicals found in the individual constituents are; ellagic acid, phyllembic acid, kaempferol, terchebin, corilagin from *P. emblica*; chebulin, chebulagic acids, chebulinic acid, chebulic acid, tannic acid, terchebin, linoleic acid from *T. chebula*; and, ellagic acid, ellagitannic acids, gallic acid, gallotannic acid, galloyl glucose, chebulagic acid, phylemblin from *T. bellirica*. The well documented active principles of *Triphala* are gallic, chebulinic, chebulagic, gallic and ellagic acids (Baliga, 2010). A complete metabolic profile of these formulations will help in ascertaining their biochemical make-up which, in turn will lead to the identification of new bio-actives. In the current study, we used a mass spectrometry-based global metabolomics approach to identify the metabolic constituents of Triphala. In addition, we predicted the potential targets of these metabolic constituents. The metabolites identified from this study will facilitate in the understanding of the mechanism of the action of this drug and aid in the identification of novel bioactives for the treatment of diseases.

Materials and Methods

Sample preparation - Triphala formulation routinely prescribed for therapeutic purposes was obtained in powder form from an Ayurvedic physician (R.P who is a co-author in this manuscript). The fruits of *Phyllanthus emblica*, *Terminalia chebula* and *Terminalia bellirica* were dried and deseeded. Equal amounts of each of the ingredients were taken and powdered. Methanolic and aqueous extracts were prepared based on previously described protocols

(Fawole *et al.*, 2012; Varma *et al.*, 2016). For methanolic extraction, 10g of Triphala powder was weighed and mixed with 100 ml of methanol to form a homogenous suspension. The suspension was kept overnight at room temperature on an orbital shaker set at 110 rpm to extract metabolites. This was followed by filtration using a filter paper (Whatman, UK. 125mm, 11 μ m, #1001-125) and the filtrate was collected. The filtrates were centrifuged at 12,000 rpm for 10 minutes at 4 °C and the supernatants were collected. The supernatant was incubated at -80°C overnight and centrifuged at 12,000 rpm for 10 minutes at 4°C. 1 ml of the extracts was then evaporated to dryness in pre-weighed micro centrifuge tubes using a SpeedVac concentrator. The dried extracts were weighed and dissolved in LC-MS grade water and stored at -80°C till further LC-MS/MS analysis. To obtain an aqueous extract, 10g of Triphala was weighed and mixed with 100 ml of sterile Milli-Q water was added and mixed manually to remove any clumps. The suspension is further mixed using an orbital shaker at 110rpm overnight at room temperature. The slurry was filtered through a Whatman filter paper and the filtrate was collected. The filtrates were then centrifuged at 12,000 rpm for 10 minutes at 4°C. 1ml of the extract was then evaporated to dryness using a SpeedVac concentrator. The dried aqueous extracts were weighed and dissolved in LC-MS/MS grade water and stored at -80°C till further LC-MS/MS analysis.

LC-MS/MS analysis - LC-MS/MS was carried out on a QTRAP 6500 mass spectrometer (SCIEX, Framingham, MA, USA) interfaced with a 1290 Infinity HPLC system (Agilent Technologies). The samples were injected using programmed autosampler onto Poroshell 120 EC-C18 analytical column (2.1mm x100mm, 2.7 μ) (Agilent Technologies, USA). The mobile phases used for the analysis included 0.1% formic acid in water (Solvent A) and 0.1% formic acid in 90% acetonitrile (Solvent B). The metabolites were eluted using the following gradient: 2% B for 1 min, 2-30% B for 9 min, 30-60% B for 1 min, 60-95% B for 2 min, 95% B for 4 min, 95-2% B for 12 s and 2% B for 3 min. The total run time was set to 20 min and the flow rate was set to 0.300 ml/min and the injection volume was set to 10 μ l. Data were acquired in both, positive and negative scan modes using the information dependent acquisition (IDA) method in the Enhanced MS (EMS) to Enhanced Product Ion (EPI) scan mode. The Analyst software (version 1.6.2,

Sciex) was used for the acquisition of data. The ESI source parameters of QTRAP 6500 included Ion Source Gas 1 (GS1) as 40 psi, Ion Source Gas 2 (GS2) as 40 psi, Curtain gas (CUR) as 35 psi, source temperature 350°C and Collisionally activated dissociation (CAD) gas at high. The ion spray voltage was set to 350 V and the declustering potential was set to 100 V in the negative as well as positive scan modes. The instrument was set to acquire m/z range 50-1000 Da.

Data preprocessing - The acquired .wiff files were converted into .mzXML format using the MSConvert GUI (64-bit) application which is part of the ProteoWizard 3.0.10325 suite (Holman *et al.*, 2014). The datasets were imported to MZmine 2.23 and subjected to data pre-processing according to previously described parameters (Lemonakis *et al.*, 2017). Briefly, the steps of pre-processing included baseline correction, mass detection, detection of chromatograms, deconvolution, deisotoping, identification of peak complexes, alignment and gap filling.

Identification of potential target metabolites - The features detected from the preprocessing steps were searched against publicly available metabolite databases to identify putative metabolites. The MS spectra of metabolites were searched in MZmine 2.23 using Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>) database with a m/z tolerance of 5ppm.

Pathway analysis - The identified metabolites were subjected to pathway analysis using Metaboanalyst 3.0 (<http://www.metaboanalyst.ca/>) (Xia *et al.*, 2015). The *Arabidopsis thaliana* (thale cress) pathway library was chosen for the analysis. The pathway algorithms used for the analysis included hypergeometric test for over representation analysis and relative-betweenness centrality for pathway topology analysis. The resulting pathway impact and -log (p) values were used to plot graphs. Graphs were plotted using Plotly (Plotly Technologies Inc. Collaborative data science. Montréal, QC, 2015. <https://plot.ly>.)

Target analysis - The protein targets for identified Triphala metabolites were predicted using "Find my Compound's Targets" tool in BindingDB (Nicola *et al.*, 2015) (<https://www.bindingdb.org/bind/chemsearch/marvin/FMCT.jsp>). SMILES IDs were fetched for all the identified compounds and used for the analysis. A similarity threshold of 0.85 was used for the prediction. Accessions were

obtained for all identifications. All the predicted proteins of human origin were used further for network analysis. Network analysis was carried out using STRING (<http://string-db.org/>) (Szklarczyk *et al.*, 2015). The parameters used for the STRING analysis included interaction sources from experiments, databases, co-expression, neighborhood, gene fusion, co-occurrence and text mining. A minimum interaction score of 0.7 or high confidence interactions were considered. K means clustering was used to cluster the network into 5 clusters.

We carried out a gene ontology analysis and visualization for all the human proteins identified from BindingDB using Cytoscape 3.6.0 (Shannon *et al.*, 2003) and the plugins ClueGO v2.5.0 (Bindea *et al.*, 2009) and CluePedia v1.5.0 (Bindea *et al.*, 2013). The CLUEGO: Functions analysis mode was used for the analysis and *Homo sapiens* was selected as the species. The ClueGO settings used included KEGG (01.03.2017) ontology, with medium network specificity, $pV \leq 0.05$, and a Kappa Score of 0.5 for GO Term/Pathway Network Connectivity. We also carried out the pathway analysis of binding partners using DAVID (<https://david.ncifcrf.gov>).

Results and Discussion

A mass spectrometry-based approach was employed to carry out the global metabolite profiling of Triphala, a traditional medicine formulation composed of *Phyllanthus emblica*, *Terminalia bellirica* and *Terminalia chebula*. The brief experimental workflow of the study is provided as **Figure 1**. A total of 1,897 metabolites were identified from both methanolic and aqueous extracts. The various identified features from Triphala are depicted as **Figure 2A**. The complete list of identified metabolites is provided in **Supplementary Table 1**. The MS/MS spectra for two of the identified compounds-rutin and 1,2,3,6-tetra-O-galloylβ-D-glucose have been provided as Supplementary Figures 1 and 2. The data for both methanolic and aqueous extracts of Triphala were acquired in both positive and negative ion modes. The comparison between these datasets is illustrated in **Figure 2B**. A total of 981 metabolites were identified from methanolic extract while 916 metabolites were identified from the aqueous extracts. Only 33 metabolites were exclusively identified from methanolic extracts while 238 metabolites were exclusively identified from aqueous extract. The rest of the 1,626 metabolites

were identified in both methanolic and aqueous extracts. Comparison of data between positive and negative ion modes revealed that 964 metabolites were identified in data acquired in the positive ion mode while 933 metabolites were identified in data acquired in the negative ion mode. There was no overlap between data acquired in positive and negative modes.

Identification of previously known metabolites of Triphala

A few studies on the determination of compounds in Triphala have been carried out using HPLC or mass spectrometry-based methods. A summary of these studies has been provided in **Table 1**. Singh and colleagues developed a high-performance liquid chromatography method for the separation and quantitation of polyphenols from Triphala (Singh *et al.*, 2008). The 5 metabolites measured included gallic acid, tannic acid, syringic acid, epicatechin and ascorbic acid. Recently a LC-MS-based method was developed to determine gallic acid, piperine and guggulsterones in Triphala guggulu (Muguli *et al.*, 2015). A study by Varma and colleagues carried out LC-MS analysis and identified gallic acid, ellagic acid, and chebulinic acid as the major constituents of Triphala (Varma *et al.*, 2016). We compared our data with previous studies and found compounds including syringic acid, chebulinic acid, epicatechin and derivatives of gallic acid and ascorbic acid to be commonly identified between the datasets. Gallic acid is a phenolic acid occurring in plants with diverse activities. It has been shown to have antioxidant activity and induce cell-mediated death (apoptosis) in promyelocytic leukemia HL-60RG (Inoue *et al.*, 1994) and B cell lymphoma cell models (Serrano *et al.*, 1998). In addition, gallic acid has been shown to have a potential therapeutic role in neurodegenerative diseases. Previous *in vitro* studies have indicated the inhibitory activity of gallic acid on the alpha-synuclein-mediated formation of amyloid fibrils suggesting its potential role as a therapeutic agent for Parkinson's disease (Liu *et al.*, 2014). In addition, it was shown to confer neuroprotection against alpha-synuclein-induced toxicity in human neuroblastoma M17 cells (Ardah *et al.*, 2014). Further, gallic acid and Triphala extract have been previously known to inhibit drug metabolizing enzymes including CYP3A4 and CYP2D6 (Ponnusankar *et al.*, 2011). In addition, it was found less likely to interact with other

co-administered drugs. Syringic acid is a phenolic compound shown to have antimitogenic and chemosensitizing in colorectal cancer cells (Abaza *et al.*, 2013). Syringic acid was also found to possess anti-angiogenic activity by decreasing expression of VEGF suggesting a potential therapeutic role in cancer (Karthik *et al.*, 2014). Further, it was found to positively influence the glycemic status in induced diabetic rats by decreasing plasma glucose and increasing plasma insulin and C-peptide levels (Muthukumaran *et al.*, 2013). In addition, it has also been found to prevent diabetic cataract in rat lenses by inhibiting aldose reductase expression and activity (Wei *et al.*, 2012). Syringic acid has also been shown to decrease hypertension in rat models through reduction of oxidative stress (Kumar *et al.*, 2012). It has also been observed to confer hepatoprotective effects in rat models with acetaminophen-induced hepatotoxicity (Ramachandran *et al.*, 2010). Chebulinic acid is an ellagitannin present in several plants. Chebulinic acid extracted from *Terminalia chebula* has been shown to have anticancer activity in HOS1 osteosarcoma cell model (Saleem *et al.*, 2002). In addition, crude extract of *T. chebula* was found to decrease cell proliferation of breast and prostate cancer cells. Chebulinic acid has also been proven to inhibit VEGF-mediated angiogenesis, thus suggesting its potential role in inhibiting tumor angiogenesis and metastasis (Lu *et al.*, 2012). Epicatechin is an antioxidant flavanoid present in several plants. It has been found to be a potential therapeutic agent for Alzheimer's disease. Epicatechin has been found to inhibit amyloid β -induced neuronal cell death (Heo *et al.*, 2005) and amyloidogenic processing (Cox *et al.*, 2015). It has also been known to enhance spatial memory in mouse models (van Praag *et al.*, 2007). Epicatechin has also been known to ameliorate oxidative damage of hippocampus caused by amyloid β 25-35 in rats (Cuevas *et al.*, 2009).

We also compared our data with data from other HPLC and LC-MS-based metabolomics studies on *Phyllanthus emblica* (Avula *et al.*, 2013; Kumar, S. *et al.*, 2017; Kumar, Sunil *et al.*, 2017; Yang *et al.*, 2012) and *Terminalia* species (Avula *et al.*, 2013; Dhanani *et al.*, 2015). This comparison led to the identification of several previously reported metabolites including 1,2,3,6-tetra-O-galloyl β -D-glucose, chebulinic acid, methyl gallate, rutin, vanillic acid, (+)-catechin, epicatechin, eriodictyol and intermediates of quercetin, cinnamic acid,

kaempferol, luteolin, quinic acid, caffeic acid and coumaric acid. From the above studies it is evident that despite of the HPLC, LC-MS or GC-MS analysis, the studies confined themselves to examining the presence and levels of the known bioactives of Triphala and did not explore to elucidating the complete metabolomic profiles. The identification of various other metabolites will help

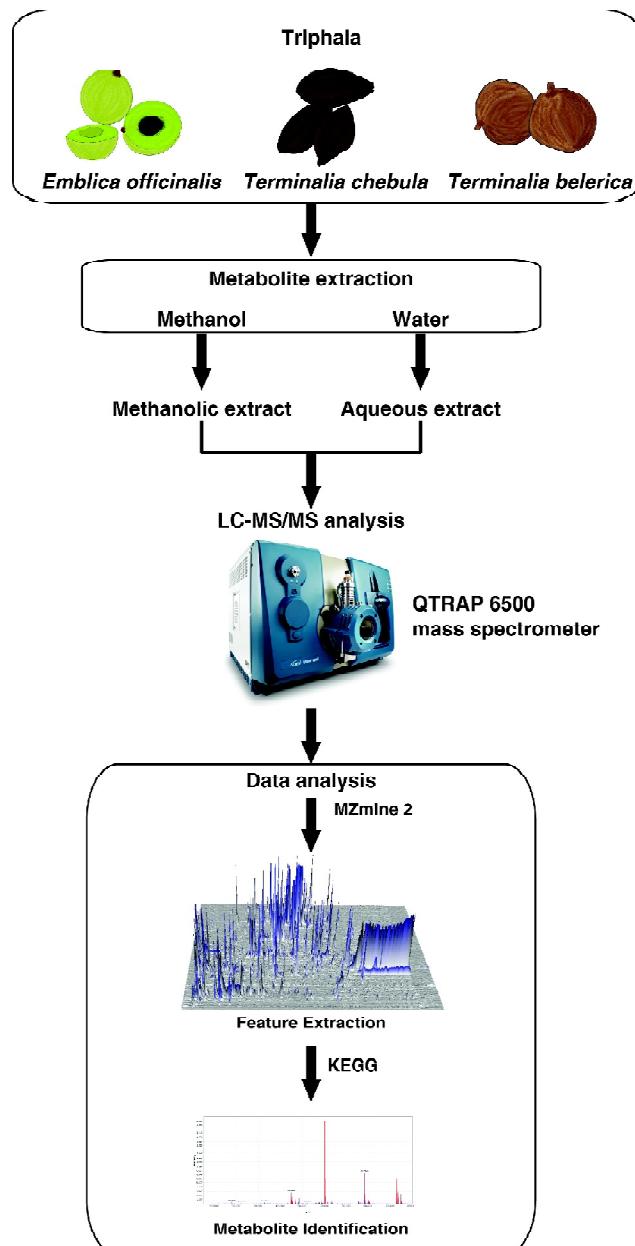


Figure 1: An illustrated view of the experimental workflow used to carry out global metabolite profiling of Triphala. Aqueous and methanolic extracts of Triphala were prepared and analyzed by LC-MS analysis using the QTRAP 6500 mass spectrometry. The mass spectrometry data obtained was processed and searched against KEGG compound database to identify metabolites using MZmine 2

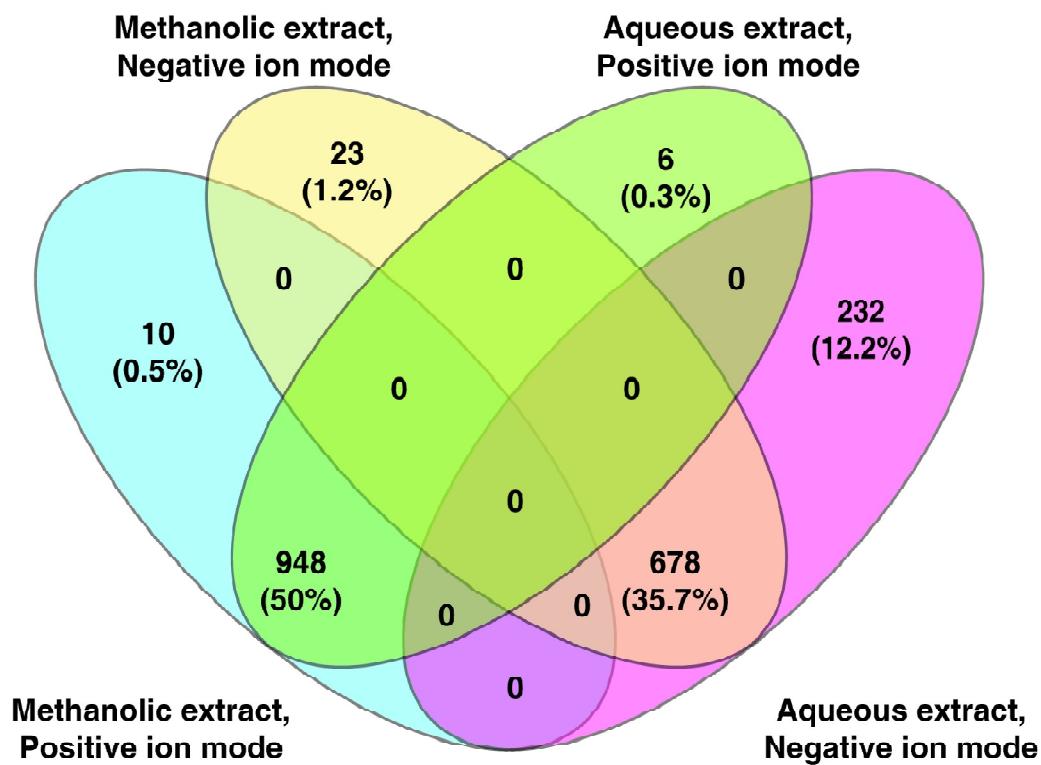
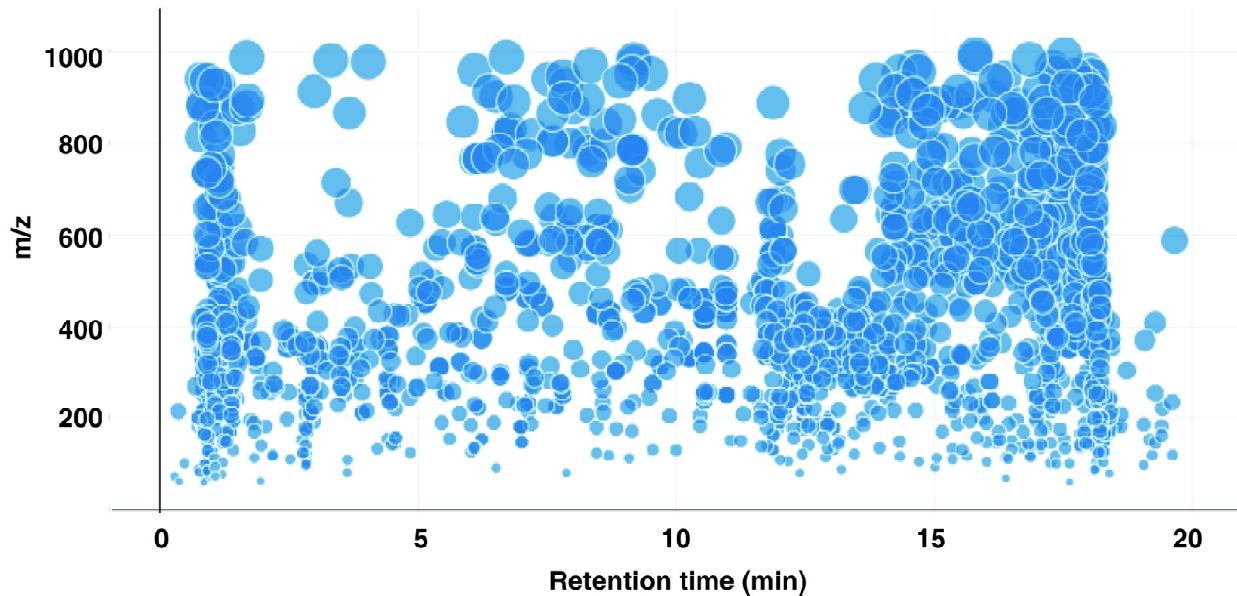


Figure 2: (A) Metabolite features identified in Triphala (B) Venn diagram depicting the overlap of identified metabolites across experiments carried out in positive and negative ion modes for aqueous and methanolic extracts of Triphala

Table 1
Previous studies on Triphala in comparison to the current study

Study	Instrumentation	Number of metabolites identified\quantified	Metabolites identified
Singh <i>et al</i> (2008) (PMID: 17879225)	HPLC	5	Ascorbic acid Tannic acid Gallic acid Syringic acid Epicatechin
Muguli <i>et al</i> (2015) (PMID: 26109777)	Electron spray ionization mass spectrometry	3	Gallic acid Piperine Guggulsterones
Varma <i>et al</i> (2016) (PMID: 26731545)	LC-MS	6	Gallic acid Ellagic acid Chebulagic acid Chebulinic acid Emblicanin A Emblicanin B GeraniinFriedelin
Current study (2017)	LC-MS	1,897	4-Coumaroylshikimate, p-Coumaroyl-CoA, Feruloyl-CoA, apigenin, quercetin 3-O-glucoside, rutin, Secologanin, 3-alpha(S)-Strictosidine, 16-Epivellosimine (For complete list refer Supplementary Table 1)

in classifying new sets of potential bioactive components and thereby help in understanding the mechanism of action of the formulation in combating various disease models.

Triphala has been reported to possess laxative properties. It has been previously shown that oral administration of *Terminalia chebula*, one of the constituents of Triphala, leads to increasing gastric emptying in rat models (Tamhane *et al.*, 1997). Several compounds are known to be present in herbal laxatives including frangula-emodin, aloe-emodin, chrysophanol and rhein (van Gorkom *et al.*, 1999). In Triphala, we identified a derivative of chrysophanol - chrysophanol 8-O-beta-D-glucoside which may be responsible for laxative activity. In addition, we also identified D-Sorbitol which has been previously attributed to possess laxative effects (Peters *et al.*, 1958).

Novel metabolites identified in this study

Several novel metabolites were identified in Triphala extract in the current study. Several classes of metabolites with potential therapeutic effects

were identified. We carried out pathway analysis for the metabolites identified in Triphala using Metaboanalyst 3.0. The impact of pathways and the top 25 pathways enriched are depicted as **Figure 3A and 3B**. The complete list of identified pathways is provided in **Supplementary Table 2**. The top five pathways found to be enriched included components of thiamine metabolism; Tyrosine metabolism; Lysine biosynthesis; Isoquinoline alkaloid biosynthesis; and Stilbenoid, diarylheptanoid and gingerol biosynthesis. The enriched pathways were found to contain several metabolites with drug activity. Some of the compounds identified from Triphala for the first time belong to metabolite classes including flavonoids, stilbenoids, diarylheptanoid and gingerolindole alkaloids.

A few flavonoids were identified in Triphala including apigenin, quercetin 3-O-glucoside and rutin in Triphala extract. Apigenin has been shown to induce cell cycle arrest in breast cancer cells through the decrease of cyclin B1 and CDK1 levels and the inhibition of MAPK phosphorylation (Yin *et al.*, 2001). It has also been seen to inhibit UV-

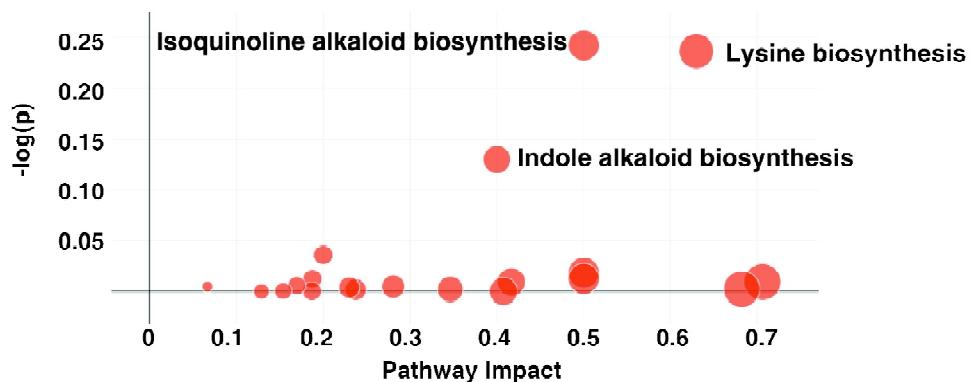
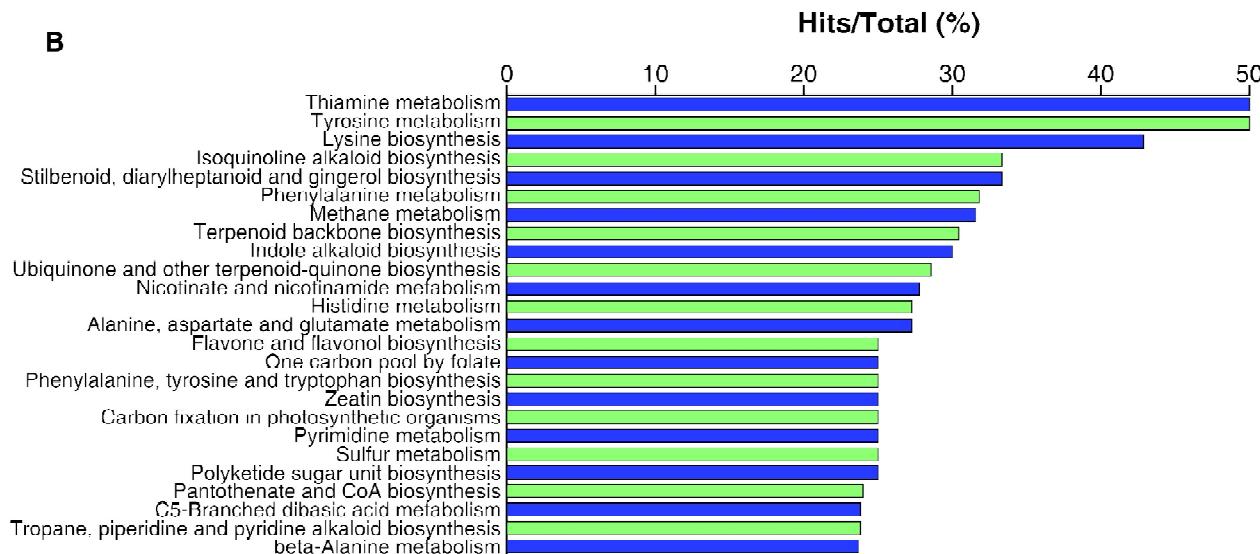
A**B**

Figure 3: (A) Graph depicting the pathway impact of enriched pathways in Triphala (B) Results of the pathway analysis in Triphala indicate the enrichment of several metabolic pathways

induced skin carcinogenesis in mice model (Birt *et al.*, 1997). Further, Apigenin has been known to induce apoptosis in prostate cancer cells (Gupta *et al.*, 2001). Quercetin 3-O-glucoside or isoquercitin has been found to have varied activities. A study by Li *et al* found that isoquercitin could bind and inhibit α -glucosidase thereby suggesting its antidiabetic activity (Li *et al.*, 2009). Isoquercitin has also been found to have neuroprotective effect protecting them from oxygen/glucose deprivation and reperfusion injury through a NF κ B and TLR4 signaling (Wang *et al.*, 2013). Rutin is a flavonoid found to have several biological activities including anti-cancer (Deschner *et al.*, 1991), anti-diabetic (Li *et al.*, 2009), anti-inflammatory (Afanas'eva *et al.*, 2001), antioxidant (La Casa *et al.*, 2000) and

neuroprotective (Tongjaroenbuangam *et al.*, 2011) properties.

Stilbenoids, diarylheptanoid and gingerol form an important class of anticancer compounds. Stilbenoids from *Vateria indica* have been shown to exhibit anticancer activity in sarcoma allograft models (Mishima *et al.*, 2003). In another study, a lignan-stilbenoid rich extract from knots of *Pinus sylvestris* was shown to exhibit antiproliferative and proapoptotic properties in human prostate cancer cell lines and xenograft animal models (Yatkin *et al.*, 2014). Diarylheptanoids are a class of plant secondary metabolites which have been studied for their anti-tumor properties. A study by Akhisa and colleagues isolated acerogenin M, a cyclic

diarylheptanoid from *Acer nikoense* and showed the anti-inflammatory and anti-tumor effects of the compound (Akihisa *et al.*, 2006). Yakuchinone A and yakuchinone B, two diarylheptanoids present in *Alpinia oxyphylla* were found to have anti-tumor properties (Chun *et al.*, 1999). In human leukemia HL-60 cell models, these compounds were found to decrease expression of epidermal ornithine decarboxylase activity and tumor necrosis factor-alpha induced by 12-O-tetradecanoylphorbol-13-acetate, a tumor promoter. Gingerols have been found to have anti-cancer activity in several cancers including triple negative breast cancer (Martin *et al.*, 2017), oral cancer and cervical cancers (Kapoor *et al.*, 2016). The Stilbenoid, diarylheptanoid and gingerols identified in Triphala include 4-Coumaroylshikimate, p-Coumaroyl-CoA and Feruloyl-CoA.

Indole alkaloids are an important class of therapeutic compounds derived from plants. This class of metabolites includes anti-cancer compounds vincristine and vinblastine (Zhu *et al.*, 2015). They also form potential leads for antidepressants with proven monoamine oxidase inhibitor activity (Dos Santos Passos *et al.*, 2013; Passos *et al.*, 2013). The indole alkaloids identified from Triphala extract include Secologanin, 3-alpha(S)-Strictosidine and 16-Epivellosimine. These compounds identified form intermediates of the indole alkaloid biosynthesis pathway (Facchini *et al.*, 2005).

Network pharmacology-based prediction of protein targets of metabolites identified from Triphala

We identified 387 potential protein targets for Triphala compounds using BindingDB. Of these 196 mapped to human proteins and the rest mapped to other species. The complete list of predicted targets of Triphala metabolites is provided in **Supplementary Table 3**. The predicted protein targets of Triphala include neuromodulatory proteins including members of the opioid receptor, 5-Hydroxytryptamine receptors, alpha adrenergic receptors, GABA receptors, glucocorticoid receptor, glutamate receptor, Nociceptin receptor and Neuropeptide Y receptor type 5. We also identified several kinases to be targets of Triphala metabolites including Aurora kinase B, AXL receptor tyrosine kinase, Mammalian target of Rapamycin (mTORC1), MAP kinase p38, PI3-kinase, Protein kinase C, Serine/threonine-protein kinase MRCK alpha, Serine/threonine-protein kinase PIM,

Tyrosine-protein kinase Lck and vascular endothelial growth factor receptor

The proteins mapping to human proteins were further analyzed using STRING to identify networks affected by Triphala (**Figure 4A**). In addition, we also carried out gene ontology analysis of protein targets of Triphala identified with BindingDB using Cytoscape (**Figure 4B**). The analysis indicated enrichment of several target pathways of Triphala including MAPK signaling pathway, VEGF signaling pathway, serotonergic synapse pathway, drug metabolism, retrograde endocannabinoid signaling and others. Correlating the results of the gene ontology analysis with the literature discussed in previous sections, it is clear that anticancer and neuromodulatory activities are some of the primary areas where Triphala may play a role. We also carried out pathway analysis with KEGG database using DAVID (**Supplementary Table 4**) and identified the enrichment of several pathways including neuroactive ligand-receptor interaction, serotonergic synapse, arachidonic acid metabolism and VEGF signaling pathways.

Interestingly, some of the potential targets of Triphala included bacterial proteins α -Carbonic anhydrase 2 (CA 2) (*Mycobacterium tuberculosis*), Anthrax Lethal Factor (LF) (*Bacillus anthracis*), Beta-lactamase ACC-4(*Escherichia coli*), Class C beta-lactamase CMY-36 (*Klebsiella pneumoniae*), Cysteine protease falcipain-3 (*Plasmodium falciparum*), Cytochrome P450 144 (CYP144) (*Mycobacterium tuberculosis*), Cytochrome P450 51(CYP51), (*Mycobacterium tuberculosis*), Falcipain-2 (*Plasmodium falciparum*) among others. We also compared our analysis with a previous analysis on binding targets of Triphala carried out by Chandran *et al.* Our results largely correlated with previous results through the identification of common targets including FLT3, AXL, GSTA1, SERPINE1, CA2, CA6, HMGCR, HGF, IGF1R, SRC, VEGFR2, CYP1B1 and GLO1 (Chandran *et al.*, 2015).

Conclusions

The data gathered from this study greatly expands the understanding of the metabolic constituents of Triphala and potential bioactive compounds. Previous studies focused on using either the total extract or the known bioactives of Triphala, in understanding its potential roles in various disease models. It is imperative to note that the metabolite make up of Triphala varies between preparation

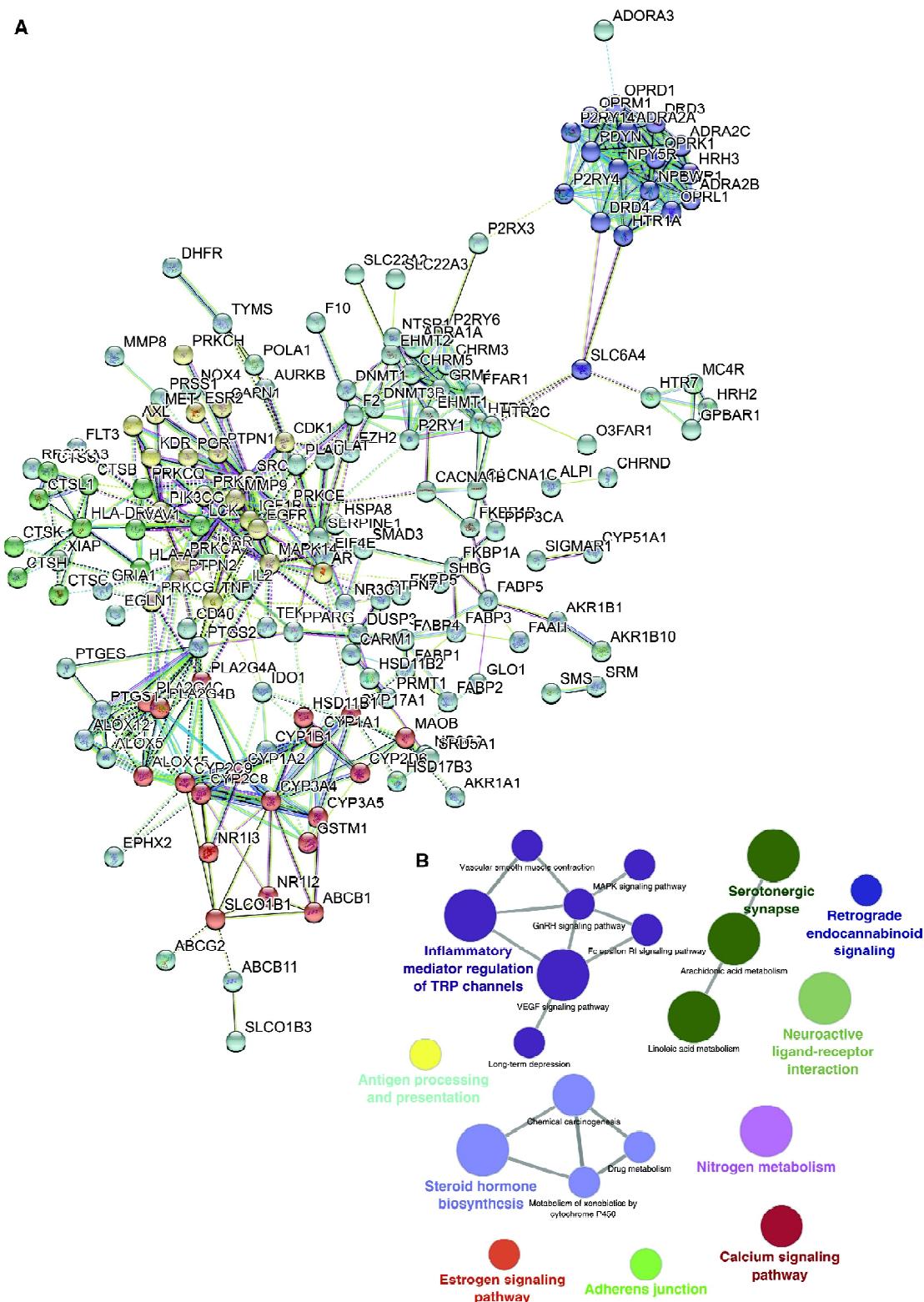


Figure 4: (A) Triphala target network. The protein targets of metabolites present in Triphala were predicted using BindingDB and network analysis was carried out for proteins of human origin using STRING. Different colors indicate clustering of proteins carried out by STRING. (B) Gene ontology analysis of predicted targets of Triphala. Gene Ontology analysis of targets of Triphala predicted using BindingDB was carried out using Cytoscape and CLUEGO. The analysis indicates enrichment of several target pathways of Triphala including MAPK signaling pathway, VEGF signaling pathway, serotonergic synapse pathway, drug metabolism, retrograde endocannabinoid signaling among others

batches, as it is an herbal formulation made from dried fruits. To ascertain its potential and activity it is thus imperative to analyze the global metabolite composition of Triphala and ensure the availability and levels of the known bioactives. This study, therefore, is a significant milestone towards complete characterization and benchmarking of traditional medicine formulations. The metabolites identified in this study need to be further investigated for their biological activities in disease models. The use of mass spectrometry-based metabolomics will open up the field of pharmacodynamics and drug repurposing. Further, this study will help in the identification of novel leads for the treatment of various diseases.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Abbreviations

HPLC, High Performance Liquid Chromatography; LC-MS, Liquid chromatography Mass spectrometry; GC-MS, Gas chromatography Mass spectrometry; GO, Gene Ontology.

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Supporting information

- Supplementary Table 1. List of metabolites identified in Triphala extract using mass spectrometry-based metabolomic analysis
- Supplementary Table 2. List of pathways identified in Triphala extract using Metaboanalyst
- Supplementary Table 3. A list of proteins predicted to interact with Triphala metabolites
- Supplementary Table 4. Results of pathway analysis of Triphala binding partners with DAVID.
- Supplementary Figure 1. Representative MS/MS spectra for rutin, a metabolite identified from Triphala.
- Supplementary Figure 2. Representative MS/MS spectra for 1,2,3,6-tetra-O-galloylβ-D-glucose, a metabolite identified from Triphala