Chromatographic and Tandem mass spectroscopic characterization of *Ayush Kwath Churnam*-an Ayurvedic immunomodulatory drug during COVID-19

Sulaiman C T (✉ slmnct@gmail.com)

Arya Vaidya Sala, Kottakkal <https://orcid.org/0000-0002-3643-6290>

Deepak M

Arya Vaidya Sala, Kottakkal

Madhavankutty T S

Arya Vaidya Sala, Kottakkal

Anandan E M

Arya Vaidya Sala, Kottakkal

Indira Balachandran

Arya Vaidya Sala, Kottakkal

Research Article

Keywords: Ayush Kwath Churna, Covid-19, Ayurveda, Immunomodulatory, HPTLC, LC/MS

Posted Date: October 6th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-85115/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Severe acute respiratory syndrome coronavirus-2, the causative agent of coronavirus disease 2019 (COVID-19) is the global health crisis. There are no existing solutions to COVID 19 as yet. *Ayush Kwath Churnam* (AKC) is an Ayurvedic medicine promoted by Ministry of AYUSH, Government of India for the enhancement of body immunity in Covid-19 scenario and promoted many AYUSH clinics all over the country with an aim of enhancing immunity of the people to resist SARS Cov-2.

Methods: In this study, chemical evaluation of AKC has been done by High Performance Thin Layer Chromatography (HPTLC) and Liquid Chromatography Mass Spectroscopic (LC-MS) analysis. Chemical characterization of AKC was carried out by Electro Spray chemical Ionization (ESI) in both positive and negative mode using Quadrupole Time-of-Flight (Q-TOF) mass spectroscopy.

Results: Data processing of molecular ions obtained by mass fragmentation led to the identification of several phytoconstituents belonging to various classes of compounds such as alkaloids, phenolics, flavonoids and isoflavones.

Conclusion: The study concluded that AKC contains variety of phytochemicals with numerous biological properties like immunomodulatory, anti-inflammatory and antioxidant activities.

1. Introduction

Coronavirus disease 2019 (COVID-19), was declared a pandemic by the World Health Organization and is a major global health concern. How to treat patients with COVID-19 effectively is a common question for health professionals worldwide. To date, therapeutic options for severe COVID-19 remain limited. Severe disease has been associated with changes in peripheral immune activity, including increased levels of pro-inflammatory cytokines that may be produced by a subset of inflammatory monocytes, lymphopenia and T-cell exhaustion [1,2]. Immunomodulatory therapy to down-regulate the cytokine storm may provide insights into the treatment of COVID-19. Natural compounds have contributed enormously to immunomodulatory therapeutics since ancient times. There are thousands of natural compounds that are known to influence the immune system by either affecting the functions of immune cells or affecting antibody secretion to control the infection and to maintain immune homeostasis. Therefore, it is essential to study the ingredient analysis of herbal preparations to discover novel promising candidates that can be used in the immunotherapeutic strategies for management of Covid-19.

Ayurveda is one of the oldest systems of medicine and the herbal drugs used in the system possess various ethnopharmacological activities such as immunostimulation, neurostimulation, anti-ageing, antibacterial, antiviral, antirheumatic, anticancer, adaptogenic, etc. Ayurveda classics have explained "*Rasayana*" drugs to enhance body resistance. They consist of a number of plants reputed to promote physical and mental health, improve defense mechanisms of the body and enhance longevity [3,4]. These

attributes are similar to the modern concept of adaptogenic agents, which are known to provide protection of the human physiological system against diverse stressors. Many Ayurvedic drugs have been claimed to possess immunomodulatory activity [5-7].

Majority of Ayurvedic formulations contain multiple botanicals as ingredient materials, some may be having minerals, metals, and ingredients of animal origin and each of these comprises a number of chemical compounds that may give the anticipated activity in combination. Polyherbal formulations show high effectiveness due to the presence of active phytochemicals which are further potentiated with synergetic interaction of active components of ingredient plants. AKC is prepared out of specified parts of four important medicinal plants such as *Ocimum tenuiflorum* L., *Cinnamomum verum* J.Presl, *Zingiber officinale* Roscoe and *Piper nigrum* L. The ingredient analysis of a Polyherbal preparation is very imperative to gain a better understanding of the chemical basis of the therapeutic potential. The present study is focused on chemical analysis of AKC using chromatographic and tandem mass spectroscopic investigations.

2. Materials And Methods

2.1 Sample Preparation

Ayush Kwath Churnam (AKC) obtained from Product Development Department, Arya Vaidya Sala, Kottakkal, Kerala, India has been taken for the study. 2g of AKC was sonicated with LC/MS grade methanol in an ultra sound bath for 20 minutes. It was then filtered through membrane filter (0.45 µm) and kept under refrigerator until HPTLC and LC/MS analysis.

2.2 Instruments and general chromatographic conditions

HPTLC analysis was performed by CAMAG HPTLC system (Switzerland). Samples were applied using CAMAG ATS 4 on aluminium backed pre-coated silica gel 60F₂₅₄ HPTLC plate (Merck India). Mobile phase was standardized as toluene, ethyl acetate and methanol in the ratio of 7:3:1. The chromatogram was developed in a saturated Twin Trough chromatographic chamber (Camag, Switzerland). The developed plate was visualized under UV at 254 nm and 366 nm and in visible light after derivetizing with Anisaldehyde sulphuric acid reagent followed by heating at 105°C for 5 minutes.

LC-MS/MS experiments were performed on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 equipped with Extend-C18 column of 1.8 µm, 2.1 x 50 mm. The MS analysis was performed using ESI in both positive and negative mode. The general conditions for mass spectrometry were: drying gas (nitrogen) flow 6 L/min; nebulizer pressure 40 psig; drying gas temperature 300°C; capillary voltage 3000 V; fragmentor volt 125V; Oct RF Vpp 750 V. The injection volume was 20 µl.

2.3 Optimization of LC/MS method

After several trail injections, the best mobile phase was fixed as gradient of methanol (A) and acidified water (B) at a constant flow rate of 0.9 ml/ min, with an increase in the volume of B%; 2-20%, 4-30%, 8-40%, 15-50%. The mass fragmentation was performed with varying collision energy 4V/ 100 DA with an offset of 6V.

3. Results

3.1 HPTLC profiling

High performance thin layer chromatographic profiling of AKC showed presence of various bands under different visualization such as UV-254, UV-366 and at visible light after derivetizing with Anisaldehyde sulphuric acid reagent. Identification of compounds like piperine (R_f 0.19), ursolic acid (R_f 0.20), linalool (R_f 0.53) and eugenol (R_f 0.65) were done on the basis of matching retardation factor (Figure 1).

Figure 1 HPTLC profiling of AKC documented at UV-254, 366 and visible light

3.2 Identification of compounds by LC/MS-MS analysis

Tandem mass spectroscopic characterization was carried out with Electrospray ionization in both positive and negative modes. The base peak chromatogram (BPC) was extracted to molecular ions with Agilent Mass Hunter software. In negative mode, BPC showed 56 molecular ion peaks and based on the relative abundance, 25 ions were further fragmented in auto ms/ms analysis by collision induced dissociation. Consistency of the fragments was confirmed by targeted ms/ms analysis with fixed collision energy. The ESI-MS fingerprint of AKC in negative mode (Figure 2, Table 1) presented the ions of: m/z 191 - quinic acid, m/z 153.0256 – 2,5-Dihydroxybenzoic acid, m/z 169.015– gallic acid, m/z 133.0179– malic acid, m/z 305.0386– gallo catechin [8-9].

Figure 2 ESI-MS- Base Peak Chromatogram of AKC in negative ionization

The fragmentation patterns of ions with m/z 353.1289 and 179.0777 are in consistent with that of caffeoylquinic acid and caffeic acid when compared with that of previous reports [10,11]. Phenolics such as 6-Hydroxy flavone (m/z 237.0538), 2-Coumaric acid (m/z 163.0499), Ferulic acid (m/z 193.0913), 2-O-caffeoylglucaric acid (m/z 371.037) and 2',6-Dihydroxy flavanone (m/z 255.245) were identified by comparing their mass fragments with that of reported values [12,13]. Compounds such as 6-Gingerol (m/z 293.346), 8-Shogaol (m/z 303.420), 8-Gingeridone (m/z 319.263) and 10-Gingeridone (m/z 347.486) might be extracted from *Zingiber officinale* and were reported earlier [14]. The ions at m/z 285.246 and 447.386 were identified as Kaempferol and Luteolin-7-Oglucoside based on their fragmentation.

Figure 3 ESI-MS- Base Peak Chromatogram of AKC in positive ionization

The ESI-MS fingerprint in positive mode (Figure 3, Table 1) presented the ions of: m/z 149.162– Cinnamic acid, m/z 457.716– Ursolic acid, m/z 449.427– kaempferol 7-O-glucoside m/z 271.257–

apigenin, 361.326-Rosmarinic acid and 595.518-Apigenin 6,8-di-glucoside The mass fragmentation patterns of these compounds are found to be in agreement with that of previous reports [9,15]. Alkaloids such as Piperine (m/z 286.1576) and piperanine (m/z 288.1687) has been reported previously from *Piper nigrum* [16,17] and are known for their immunomodulatory properties [18,19].

Table 1 ESI-LC-MS/MS analysis of AKC

Sl No.	m/z	MS/MS	Tentative Identification	Molecular formula	Ionization mode
1	191.0568	173.10	Quinic acid	C ₇ H ₁₁ O ₆	Negative
2	153.0256	109.03	2,5-Dihydroxybenzoic acid	C ₇ H ₆ O ₄	Negative
3	169.015	125.02	Gallic acid	C ₇ H ₆ O ₅	Negative
4	133.0179	115	Malic acid	C ₄ H ₆ O ₅	Negative
5	305.0386	225.02	Gallo catechin	C ₁₅ H ₁₄ O ₇	Negative
6	353.1289	191.05, 179.12	Caffeoylquinic acid	C ₁₆ H ₁₈ O ₉	Negative
7	179.0777	162,135.08	Caffeic acid	C ₉ H ₈ O ₄	Negative
8	237.0538	193.06	6-Hydroxy flavone	C ₁₅ H ₁₀ O ₃	Negative
9	163.0499	119.05	2-Coumaric acid	C ₉ H ₈ O ₃	Negative
10	193.0913	149.1	Ferulic acid	C ₁₀ H ₁₀ O ₄	Negative
11	371.037	353.02,191.02	2-O-caffeoylglucaric acid	C ₁₅ H ₁₆ O ₁₁	Negative
12	255.245	209.12	2',6-Dihydroxyflavanone	C ₁₅ H ₁₂ O ₄	Negative
13	293.346	193.21	6-Gingerol	C ₁₇ H ₂₆ O ₄	Negative
14	285.246	151.20, 135.26	Kaempferol	C ₁₅ H ₁₀ O ₆	Negative
15	303.420	167.36	8-shogaol	C ₁₉ H ₂₈ O ₃	Negative
16	319.263	193.16,	8-Gingerdione	C ₁₉ H ₂₈ O ₄	Negative

		182.83			
17	347.486	210.46, 152.41	10-gingerdione	C ₂₁ H ₃₂ O ₄	Negative
18	447.386	284.32, 240.10	Luteolin-7-O-glucoside	C ₂₁ H ₂₀ O ₁₁	Negative
19	149.162	133.26	Cinnamic acid	C ₉ H ₈ O ₂	Positive
20	457.716	411.82	Ursolic acid	C ₃₀ H ₄₈ O ₃	Positive
21	449.427	287.26	Kaempferol 7-O-glucoside	C ₂₁ H ₄₂ O ₁₁	Positive
22	271.257	253.36, 225.17	Apigenin	C ₁₅ H ₁₀ O ₅	Positive
23	361.326	198.20, 180.42	Rosmarinic acid	C ₁₈ H ₁₆ O ₈	Positive
24	595.518	413.42	Apigenin 6,8-di-C-glucoside	C ₂₇ H ₃₀ O ₁₅	Positive
26	286.1576	201.20, 173.51	Piperine	C ₁₇ H ₁₉ NO ₃	Positive
27	288.1684	161.23, 135.18	Piperanine	C ₁₇ H ₂₁ NO ₃	Positive

4. Discussion

Various traditional medicines have been found to modulate various components of innate and acquired immune system. The use of herbal products as immunomodulators has a long history and the ancient times treatment of many diseases were based on modulating the immune system using herbal medicines [20]. The main target of the immunomodulatory natural products is primarily there action on the macrophages which play a key role in the generation of an immune response. The therapeutic potential

of immunomodulatory compounds from herbal products and the Ayurvedic concepts of preventive health care have been reported earlier [21]. The present scenario of Covid-19 pandemic also indicates that natural products may be lead compounds, for the development and the discovery of new therapeutic agents for the management of Covid-19.

Ayush Kwath Churnam is recommended by Ministry of AYUSH, Government of India for immunity enhancement in the present situation of pandemic Covid-19. The metabolite profiling of AKC depicted presence of 27 identified phytochemicals belonging to different class of compounds such as alkaloids, phenolics, flavonoids and phenolic glycosides. These compounds are reported to possess numerous biological activities including immunomodulatory, anti-inflammatory and anti-oxidant properties. Most of the compounds identified from AKC are polyphenolics in nature. Various studies have shown the immunomodulatory effects of polyphenols through different mechanisms such as reformation of composition and level of immunoglobulins, populations of immune cells [22,23]. Many studies have been reported regarding the utility of natural products as immunomodulatory agents to enhance host responses and to reduce damage to the human body. Immunomodulatory effect of caffeic, ferulic, and p-coumaric acids has been reported previously [24]. ESI negative mode ionization showed presence of many compounds from *Z. officinale* including 6-Gingerol, 8-shogaol, 8-Gingerdione and 10-gingerdione which have been reported to possess immunomodulatory properties [25,26]. In ESI positive ionization, alkaloids such as Piperine and Piperanine were detected from AKC and that might have extracted from the ingredient plant *Piper nigrum*. The immunomodulatory potential of piperine has been reported earlier [27]. Piperine was reported to inhibit proliferative response induced by lipopolysaccharide (LPS) and immunoglobulin α -IgM antibody and resulted in inhibition of IgM antibody secretion and reduced expression of cluster of differentiation CD8612 [28].

Conclusion

The current COVID-19 pandemic is a global unprecedented challenge for human health and researchers are desperately seeking a proven cure for it. When the conventional drugs such as lopinavir, ritonavir, chloroquine, and hydroxychloroquine are not as effective as anticipated, research on traditional herbal medicine is a viable strategy for Covid-19 management and that should be scientifically validated. The present study concluded that the *Ayush Kwath Churna*, a popular Ayurvedic drug premeditated during Covid-19 contains many active molecules with known therapeutic potentials like immunomodulatory, anti-inflammatory and anti-oxidant activities and it will be beneficial to acquire immunity against the severe conditions due to the Coronavirus-2 attack.

Declarations

Competing interests: The authors declare no competing interests.

Funding: This work was supported by Navajbhai Ratan Tata Trust, Mumbai, India.

References

1. Wilk AJ, Rustagi A, Zhao NQ, Roque J, Martínez-Colón GJ, McKechnie JL, Ivison G T, Ranganath T, Vergara R, Hollis T, Simpson LJ, Grant P, Subramanian A, Rogers AJ, Blish CA. A single-cell atlas of the peripheral immuneresponse in patients with severe COVID-19. Nat Med 2020.
<https://doi.org/10.1038/s41591-020-0944-y>.
2. Zhao M. Cytokine storm and immunomodulatory therapy in COVID-19: Role of chloroquine and anti-IL-6 monoclonal antibodies, Int J Antimicrob Agents. doi: 10.1016/j.ijantimicag.2020.105982.
3. Balasubramani SP, Venkatasubramanian P, Kukkupuni SK, Plant-based Rasayana drugs from Ayurveda. Chin. J. Integr. Med. 2011; 17: 88–94.
4. Tiwari R, Latheef SK, Ahmed I, Iqbal HMN, Bule MH, Dhama K, Samad HA, Karthik K, Alagawany M, Abd El-Hack ME, Yattoo MI, Farag MR., Herbal Immunomodulators - A Remedial Panacea for Designing and Developing Effective Drugs and Medicines: Current Scenario and Future Prospects, Curr Drug Metab 2018; 19: 264-301.
5. Kumar S, Kamboj J, Suman, Sharma S. Overview for Various Aspects of the Health Benefits of *Piper Longum* Fruit J. of Acup and Meri Stud, 2011; 4: 134-140.
6. Rege NN, Thatte UM, Dahanukar SA, Adaptogenic properties of six rasayana herbs used in Ayurvedic medicine. Phytother Res 1999; 13: 275–291.
7. Kuttan G. Immunomodulatory effect of some naturally occuring sulphur-containing compounds. J Ethnopharmacol 2000; 72: 93–99.
8. Medina RIC, Segura-Carretero A, Fernandez-Gutierrez A. Use of high-performance liquid chromatography with diode array detection coupled to electrospray-Q-time-of-flight mass spectrometry for the direct characterization of the phenolic fraction in organic commercial juices. J Chromatogr A. 2009; 1216: 4736–4744.
9. Sulaiman CT, George S, Thushar KV, Balachandran I. Phenolic characterization of selected *Salacia* species using LC-ESI-MS/MS analysis, Nat. Prod. Res. 2014; 28: 1021-1024.
10. Seeram NP, Lee R, Scheuller S, Heber D. Identification of phenolic compounds in strawberries by liquid chromatography electrospray ionization mass spectroscopy. Food Chem. 2006; 97: 1–11.
11. Sulaiman CT, Balachandran I. LC/MS characterization of phenolic antioxidants of Brindle berry (*Garcinia gummi-gutta* (L.) Robson), Nat. Prod. Res. 2017; 31: 1191-1194.
12. Plazonic A, Bucar F, Males Z, Mornar A, Nigovic B, Kujundzic N. Identification and quantification of flavonoids and phenolic acids in burr parsley (*Caucalis platycarpus*), using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry, Molecules 2009; 14: 2466–2490.
13. Sulaiman CT, Balachandran I. Chemical Profiling of an Indian Herbal Formula Using Liquid Chromatography Coupled with Electro Spray Ionization Mass Spectrometry, Spectrosc. Lett. 2015; 48: 222-226.

14. Tao YI, Li W, Liang W, Van Breemen R.B. Identification and Quantification of Gingerols and Related Compounds in Ginger Dietary Supplements Using High Performance Liquid Chromatography-Tandem Mass Spectrometry, J Agric Food Chem. 2009; 57: 10014–10021.
15. Chaudhary A, Sharma S, Mittal A, Gupta S, Dua A. Phytochemical and antioxidant profiling of *Ocimum sanctum*. J Food Sci Technol 2020. <https://doi.org/10.1007/s13197-020-04417-2>.
16. Parthasarathy VA, Chempakam B, Zachariah TJ. Chemistry of spices. London: 2008. CABI.
17. Kumar PV, Kuttan R, Kuttan G.. Effect of “Rasayanas” a herbal drug preparation on cell-mediated immune responses in tumor bearing mice. Indian J. Exp Biol 1999; 37: 23.
18. Meghwa M, Goswami T K. *Piper nigrum* and Piperine: An Update, Phytother Res . 2013; 27: 1121-30.
19. Bernardo AR., da Rocha JDB, de Lima MEF, Ricardo DD, da Silva LHP, Peçanha LMT, Danelli MDGM. Modulating effect of the piperine, the main alkaloid from *Piper nigrum*, on murine B lymphocyte function. Braz. J. Vet. Med 2015; 37: 209–216.
20. Rates SMK. Plants as source of drugs. Toxicon, 2001; 39: 603-613.
21. Jatawa AS, Paul R, Tiwari A. Indian Medicinal Plants: A Rich Source of Natural Immuno-Modulator. Int. J. of Pharmacol, 2011; 7: 198-205.
22. Narayan C, Kumar A. Antineoplastic and immunomodulatory effect of polyphenolic components of *Achyranthes aspera* (PCA) extract on urethane induced lung cancer in vivo. Mol Biol Rep. 2014; 41: 179-191.
23. Sobhani M, Farzaei MH, Kiani S, Khodarahmi R.. Immunomodulatory; Anti-inflammatory/antioxidant Effects of Polyphenols: A Comparative Review on the Parental Compounds and Their Metabolites, Food Reviews Int. 2020. DOI:10.1080/87559129.2020.1717523.
24. Jaziri SK, Bzeouich IM, Krifa M, Nasr N, Ghedira K, Ghedira LC. Immunomodulatory and cellular antioxidant activities of caffeic, ferulic, and p-coumaric phenolic acids: a structure–activity relationship study Drug and Chemical Toxicology, DOI: 10.1080/01480545.2016.1252919.
25. Qian-Qian Mao, Xiao-Yu Xu, Shi-Yu Cao, Ren-You Gan,, Harold Corke, Trust Beta, Hua-Bin Li, Bioactive Compounds and Bioactivities of Ginger (*Zingiber officinale* Roscoe), Foods. 2019; 8(6): 185.
26. Amri, M, Touil-Boukoffa C. In vitro anti-hydatic and immunomodulatory effects of ginger and [6]-gingerol. Asian Pac J Trop Med. 2016; 9: 749-756.
27. Bezerra DP, de Castro FO, Alves APNN, Pessoa C, de Moraes MO, Silveira ER, Lima MAS, Elmiro FJM., de Alencar NMN, Mesquita RO et al. In vitro and in vivo antitumor effect of 5-FU combined with piplartine and piperine. J. Appl. Toxicol. 2008; 28: 156–163.
28. Bernardo AR, da Rocha JDB, de Lima MEF, Ricardo DD, da Silva LHP, Peçanha LMT, Danelli MDGM. Modulating effect of the piperine, the main alkaloid from *Piper nigrum* Linn., on murine B lymphocyte function. Braz. J. Vet. Med. 2015; 37: 209–216.

Figures

Figure 1

HPTLC profiling of AKC documented at UV-254, 366 and visible light

Figure 2

ESI-MS- Base Peak Chromatogram of AKC in negative ionization

Figure 3

ESI-MS- Base Peak Chromatogram of AKC in positive ionization