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Research Article **Open Access**

Investigating the Efficiency of Simple Aqueous Extract of *Nigella Sativa* Activity Against *Serratia Marcescens* Bactria

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ABSTRACT

Nigella sativa seeds is considered as a very effective treatment for a variety of human diseases in traditional medicine worldwide with low human toxicity. *Serratia marcescens* pathogenic species, a Gram-negative, opportunistic bacteria, are widespread and distributed in the human environment with considered resistance to different types of antibiotics. Extraction was performed on 12.5 gm. /150 ml of *Nigella* seeds using rotary evaporator. Different time periods for extraction were applied (1-4 hours) with different temperature ranges of 50 °C to 60 °C performed separately for each time period. The 3 and 4 hours extraction period at 60 °C for seeds was effective against *Serratia marcescens* with inhibition zone of 11.18 and 12.27 mm respectively. *Nigella* seeds could be a promising inhibitor with enhanced activity against *Serratia marcescens*. Water extraction was efficient and effective at 60 °C, with extraction period of 2-3 hours for seeds. No activity recorded below this temperature.

KEYWORDS: *Nigella Sativa; Serratia marcescens*; water *Nigella* Extract activity; Treatment of *Serratia marcescens* with nigella sativa.

INTRODUCTION

Seeds of *Nigella sativa* or black cumin, commonly known as black seed, have been traditionally used in treatment of headache, abdominal pain (i.e. colon, pancreatic, liver, lung, fibrosarcoma, and prostate tumor), coughs diarrhea, lipedema, asthma, rheumatism and other different diseases [1,2]. The seeds of this plant extracts are well investigated, both aqueous and oil extraction methods and have shown antioxidant, anti-inflammatory, anticancer, analgesic as well as antimicrobial activities [3-5]. Administration of *Nigella sativa* extract was safe and had no notable side effects on liver, kidneys, or gastrointestinal tract [6,7]. Qidwai *et al*. and others recorded that the administration of *Nigella sativa* seeds have no effect on serum enzyme, alanine aminotransferase (ALT), and the serum creatinine (Cr) concentration with potential treatment effect [8-10].

Different herbal extraction techniques were used (purification and fractionation) for effective collection of metabolic products. However, extraction conditions (temperature, solvents, agitation speed etc.) may enhance extraction of active ingredients [11].

Serratia marcescens pathogenic species are opportunistic Gram-negative bacteria also considered as tribe Klebsielleae [12]. It is widely distributed in the environment and can pose a significant problem when found in the respiratory, digestive, and urinary tracts in human. Healthcare workers, as well as other individuals are at risk of exposure to *Serratia marcescens* [13]. Intensive care units (ICUs) at hospitals can develop epidemics of infection with these bacteria since it is found in medical and laboratory equipment,

medications, blood products, antiseptics solution, lotions, and toilets [14,15].

Infections occurring by *Serratia marcescens* are difficult to cure. This difficulty might be as a result of its attribution to resistance to different antibiotics, including ampicillin and first and second generation's cephalosporin's [16,17]. Aminoglycosides provided good activity against *Serratia marcescens*, but resistant strains have-also been reported recently by researchers [18]. Other researchers reported an endocarditis caused by a ciprofloxacin-resistant strain of *Serratia marcescens* in which was isolated from blood culture taken from a peripheral vein and the Hickman line [19,20].

The aim of this research is to evaluate the biological activity of a simple aqueous extract of *Nigella sativa* on *Serratia marcescens* bacteria. Moreover, investigate the minimum temperature and time to collect potential aqueous extract of *Nigella* seeds.

This extract could provide a powerful treatment against *Serratia marcescens* bacteria with low cost extraction methods. To the time of writing this manuscript, no similar studies focus on the biological ability of *Nigella sativa* aqueous extract on *Serratia marcescens*.

MATERIALS AND METHODS

All the glassware had been washed with distilled and deionized water and dried using a hot air oven before use. Mueller Hinton Agar was obtained from HiCrome™ Bulgaria [21, 22]. Seeds of *Nigella sativa* were purchased from commercial market. All other chemicals and reagents used in this research are with high purity for analytical purposes.

Extraction of Nigella sativa seeds

12.5 gm of well washed seed samples were extracted in 150 ml of water using rotary evaporator at 50 °C and 60 °C separately for periods 1, 2, 3 and 4 hours, respectively for each selected temperature.

UV-Vis Spectra Analysis

The extract was well filtered; scanning was performed by using shimadzu UV-VIS spectrophotometer (UV-1650 pc) with scanning ranged from 200-700 nm.

Antibacterial Assay

Serratia marcescens bacteria were grown in Mueller Hinton Agar. The bacteria were inoculated in the LB medium in an incubator thermostat for 6 h at 37^0C. One milliliter of bacterial inoculum was added to 9 mL 0.9 % normal saline and diluted to 10^6 cfu mL^{-1} (colony forming unit, cfu), then inoculated into LB broth for 24 hour at 37^0C.[23] Well agar diffusion method was used to detect the inhibition zones in sterile molten Mueller Hilton agar with *Nigella sativa* extract. Then plates were incubated at 37 °C for 24 h with the different prepared concentrations of *Nigella sativa* extraction conditions [24, 25].

The aqueous extraction in this work was performed in relatively low temperature to avoid overheating or decomposition of organic compounds during the extraction process. Nevertheless, this work was designed to apply aqueous extraction to avoid any toxicity, interference of solvents, simplicity, and other complications of organic solvents [26].

RESULTS AND DISCUSSION

Aqueous herbal extraction one of the most familiar methods for medicinal herbal active ingredients administration. The collected results predicted a biological activity of extracted solution on

Serratia marcescens bacteria with diameters of inhibition zone 11.18 and 12.27 mm by applying extractions condition of three to four hours respectively at 60 °C. The inhibition zone is represented in Figure 1. However, lowering of extraction temperature and time periods eliminates its biological activity on *Serratia marcescens* bacteria. The results shows powerful effects of *Nigella sativa* seeds watery extract on bacteria without reach a drastic or complex biochemical extraction process [27-29].

The UV-VIS spectrum reported an increase of extracted compounds with increased temperature. The extract spectrum shows an overlap of detection peaks which could be

attributed to the wide range of chemical compounds released during extraction process from seeds as well as efficiency of the applied method [30-31].

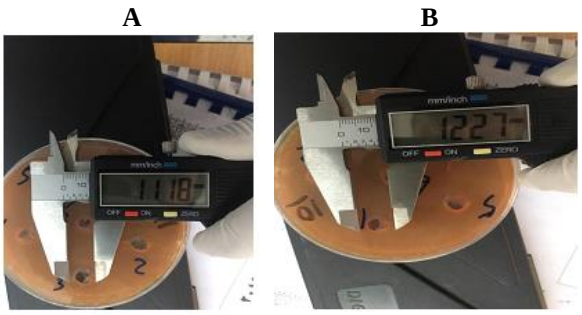

Figure 1. A- represents inhibition of extraction at 50 °C (11.18 mm), B- the inhibition of extraction on 60 °C (12.27 mm).

The most active compounds detected of *Nigella sativa* extract are the thymoquinone, dithymoquinone, thymohydroquinone, p-cymen, corvacrol, thymol and other phenolic derivatives. All extract compounds collectively observed reduce inflammation process, ontogenesis, and antitumor and antioxidant activity [32-34]. *Nigella sativa* was observed to induce antitumor effects in lung, breast, multiple myeloma, pancreatic, as well as gastrointestinal cancers. [35, 36] However, these active gradients were observed lethal on *Serratia marcescence* bacteria growth. On the other hand, other researchers observe significant increase in bacterial killing effect of *Nigella sativa* extract with added nonmaterial [37, 38].

Efflux pumps are transport protein in bacteria associated with elimination of toxic molecules from cell to external environment, moreover, this mechanism play essential role in reducing bacteria killing and develop bacteria resistance. The killing mechanism of *Nigella Sativa* extract on *Serratia marcesence* could be involved directly in the effect on cell membrane efflux pumps, which reduce bacteria resist and survive [39,40].

CONCLUSIONS

In conclusion: Water extraction for four hours at 60 °C is considered as potential technique for extraction active chemical ingredients of *Nigella sativa* seeds. The selected method in this research provided simple extraction procedure with high yield of active ingredients with reduces degradation of organic molecules result from extraction process. The selected *Nigella sativa* extraction method at pervious conditions was a very effective inhibitor to *Serratia marcescens* bacteria. From research data, *Nigella Sativa* extract may be help in treatment of *Serratia marcescens* infections. The extraction method was low cost, simple and sophisticated. However, our data provided new addition antibacterial activity to *Nigella Sativa*. We strongly recommended that more investigation of *Nigella Sativa* extract alone or mixed with other material can apply to another types of resist bacteria as well as evaluated its pharmacological action on untreated or chronic diseases [41-45].

REFERENCES

[1] Ibrahim RM, Hamdan NS, Mahmud R, Imam MU, Saini SM, Rashid SN, *et al*. A randomised controlled trial on hypolipidemic effects of *Nigella Sativa* seeds powder in menopausal women. J Transl Med. 2014;12:82. DOI: 10.1186/1479-5876-12-82

[2] Ibrahim RM, Hamdan NS, Ismail M, Saini SM, Abd Rashid SN, Abd Latiff L, *et al*. Protective effects of *Nigella sativa* on metabolic syndrome in menopausal women. Adv. Pharm Bull. 2014; 4(1):29-33. DOI: 10.5681/apb.2014.005

[3] Tavakkoli A, Ahmadi A, Razavi BM, Hosseinzadeh H. Black seed (*Nigella sativa*) and its constituent thymoquinone as an anti-dote or a protective agent against natural or chemical toxicities. Iran J Pharm Res. 2017; 16:2-23. PMCID: PMC5963642

[4] Tavakkoli A, Mahdian V, Razavi BM, Hosseinzadeh H. Review on clinical trials of black seed (*Nigella sativa*) and its active constituent, thymoquinone. J Pharmacopuncture 2017; 20:107-111. DOI: 10.3831/KPI.2017.20.021.

[5] Havakhah S, Sadeghnia HR, Mosa-Al-Reza Hajzadeh NM, Roshan SS, Hosseinzadeh H, Mohareri N, et al. Effect of *Nigella sativa* on ischemia-reperfusion induced rat kidney damage. Iran J Basic Med Sci 2014; 17:986-992. PMCID: PMC4387234

[6] Piras A, Rosa A, Marongiu B, Porcedda S, Falconieri D, Dessi MA, et al: Chemical composition and in vitro bioactivity of volatile and fixed oils of *Nigella sativa* L extracted by supercritical carbon dioxide. Industrial Crops and

Products 2013; 46(1): 317-323. DOI: 10.1016/j.indcrop.2013.02.014

[7] M. Akram Khan., M. Afzal, Chemical composition of *Nigella sativa* Linn: Part 2 Recent advances,J. Inflammopharmacology Experimental and Therapeutic Studies, June 2016, Volume 24, Issue 2–3, pp 67–79. DOI: 10.1007/s10787-016-0262-7

[8] Qidwai W, Hamza HB, Qureshi R, Gilani A. Effectiveness, safety, and tolerability of powdered *Nigella sativa* (kalonji) seed in capsules on serum lipid levels, blood sugar, blood pressure, and body weight in adults: results of a randomized, double-blind controlled trial. J Altern Complement Med. 2009; 15(6):639-44. DOI: 10.1089/acm.2008.0367,

[9] Amin B, Hosseinzadeh H. Black cumin (*Nigella sativa*) and its active constituent, thymoquinone: an overview on the analgesic and anti-inflammatory effects. Planta Med 2016; 82:8-16. 38. DOI: 10.1055/s-0035-1557838

[10] Mollazadeh H, Afshari AR, Hosseinzadeh H. Review on the potential therapeutic roles of *Nigella sativa* in the treatment of patients with cancer: involvement of apoptosis-black cumin and cancer. J Pharmacopuncture 2017; 20:158-172. doi: 10.3831/KPI.2017.20.019.

[11] Amalia T., Bogi P., Noor H., Study of Antimicrobial Activity of Black Cumin Seeds (*Nigella sativa* L.) Against Salmonella typhi In Vitro, Journal of Medical & Surgical J Pathology, Utami, et al., J Med Surg Pathol, 2016, 1:3, DOI:10.4172/2472-4971.1000127.

[12] Crivaro V, Bagattini M, Salza MF, Raimondi F, Rossano F, Triassi M, et al. Risk factors for extended spectrum beta lactamase producing *Serratia marcescens* and Klebsiella pneumoniae acquisition in a neonatal intensive care unit. J Hosp Infect. 2007; 67(2):135–41. DOI: 10.1016/j.jhin.2007.07.026

[13] Adamson V, Mitt P, Pisarev H, Metsvaht T, Telling K, Naaber P, et al. Prolonged outbreak of *Serratia marcescens* in Tartu University Hospital: a case control study. B M J Infect Dis. 2012 Oct; 12 (1) :281. DOI: 10.1186/1471-2334-12-281.

[14] Cullen MM, Trail A, Robinson M, Keaney M, Chadwick PR. *Serratia marcescens* outbreak in a neonatal intensive care unit prompting review of decontamination of laryngoscopes. J. Hosp Infect 2005; 59: 68-70. DOI: 10.1016/j.jhin.2004.08.003

[15] Das S, Sheorey H, Taylor HR, Vajpayee RB. Association between cultures of contact lens and corneal scraping in contact lens related microbial keratitis. Arch Ophthalmol 2007; 125: 1182-1185. DOI:10.1001/archopht.125.9.1182

[16] Mohtashami R, Huseini HF, Heydari M, Amini M, Sadeqhi Z, Ghaznavi H, et al. Efficacy and safety of honey based formulation of Nigella sativa seed oil in functional dyspepsia: a double blind randomized controlled clinical trial. J. Ethnopharmacol. 2015; 175: 147-52. DOI: 10.1016/j.jep.2015.09.022.

[17] Barakat EMF, El Wakeel LM, Hagag RS. Effects of *Nigella sativa* on outcome of hepatitis C in Egypt. World J Gastroenterol. 2013; 19 (16): 2529-36. DOI: 10.3748/wjg.v19.i16.2529

[18] R. Coria-Jiménez and C. Ortiz-Torres, Aminoglycoside resistance patterns of *Serratia marcescens* strains of clinical origin, J. Epidemiol Infect. 1994 Feb; 112(1): 125–131. DOI: 10.1017/S0950268800057484.

[19] Traub WH, and Fukushima PI., Nonspecific aminoglycoside cross-resistance of Serratia *marcescens*, Chemotherap. 1979; 25 (4): 204-13. DOI: 10.1159/000237841

[20] Yohei Doi, Keiko Yokoyama, Kunikazu Yamane, et.al., Plasmid-Mediated 16S rRNA Methylase in *Serratia marcescens* Conferring High-Level Resistance to Aminoglycosides, antimicrobial agent and microbiology, 2004, 10. 1128/AAC. 48.2. 491-496. DOI**:** 10.1128/AAC.48.2.491-496.2004

[21] Yeon-Joon Park, Seungok Lee, Jin Kyung Yu, et.al. Co-production of 16S rRNA methylases and extended-spectrum β-lactamases in AmpC-producing Enterobacter cloacae, Citrobacter freundii and *Serratia marcescens* in Korea, J. Antimicrobial Chemotherapy, Volume 58, Issue 4, October 2006, Pages 907–908. DOI: 10.1093/jac/dkl317.

[22] J. C. Lapenda, P. A. Silva, M. C. Vicalvi, K. X. F. R. Sena, et. al. Antimicrobial activity of prodigiosin isolated from *Serratia marcescens* UFPEDA 398, World Journal of Microbiology and Biotechnology February 2015, Volume 31, Issue 2, pp 399–406. DOI: 10.1007/s11274-014-1793-y

[23] Panel C., Arcelloni B., Comuzzi R, et.al., Quantification of gentamicin in Mueller–Hinton agar by high-performance liquid chromatography., Journal of Chromatography B: J. Biomedical Sciences and Applications., Volume 753, Issue 1, 25 March 2001, Pages 151-156. DOI: 10.1016/S0378-4347(00)00460-6

[24] Karuppusamy S, Rajasekaran KM. High throughput antibacterial screening of plant extracts by resazurin redox with special reference to medicinal plants of Western Ghats. Glob J Pharmacol. 2009; 3 (2):63–68. ISSN 1992-0075

[25] Mansi KMS. Effects of oral administration of water extract of Nigella sativa on serum concentrations of insulin and testosterone in alloxan-induced diabetic rats. Pak J. Biol. Sci., 2005; 8: 1152-1156.ISSN 1028-8880.

[26] Kokoska L, Havlik J, Valterova I, Sovova H, Sajfrtova M, Jankovska I: Comparison of chemical composition and antibacterial activity of *Nigella sativa* seed essential oils obtained by different extraction methods. J. Food Prot. 2008, 71: 2475-2480. DOI: 10.4315/0362-028X-71.12.2475.

[27] M.A. Ali, M.A. Sayeed, M.S. Alam, M.S. Yeasmi n, A. M. Khan, I. I. MuhamadCharacteristics of oils and nutrient contents of *Nigella sativa*Linn. and *Trigonella foenum-graecum* seeds, Bull. Chem. Soc. Ethiop.,2012, 26, pp. 55-64. DOI: 10.4314/bcse.v26i1.6.

[28] H.J. Harzallah, E. Noumi, K. Bekir, A. Bakhrouf, T. MahjoubChemical composition, antibacterial and antifungal properties of Tunisian *Nigella sativa* fixed oil., Afr. J. Microbiol. Res.,2012, 6, pp. 4675-4679. DOI: 10.5897/AJMR11.1073

[29] M. M. K. Ali*, M. A. Hasan and M. R. Islam, Influence of Fertilizer Levels on the Growth and Yield of Black Cumin (Nigella sativa L.), A Scientific Journal of Krishi Foundation, The Agriculturists 13(2): 97-104(2015), DOI: DOI: 10.3329/agric.v13i2.26596.

[30] Aftab A, Asif H. Mohd, M. et. al., A review on therapeutic potential of *Nigella sativa*: A miracle herb., Asian Pacific Journal of Tropical Biomedicine may 2013, Volume 3, Issue 5, Pages 337-352. DOI: 10.1016/S2221-1691(13)60075-1

[31] Selin I., Murat K., Sinem A. quantitative Analysis of Thymoquinone in *nigella sativa.* (Black cumin) seeds and commercial seed oils and seed oil capsules from turkey, J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 2017, 41(1): 34-41.

[32] Sethi G, Ahn KS, Aggarwal BB. Targeting nuclear factor-kappa B activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis. Mol Cancer Res. 2008;6 (6):1059-70. DOI: 10.1158/1541-7786.MCR07-2088. Erratum in: Mol Cancer Res. 2018;16 (9): 1441. DOI: 10.1158/1541-7786.MCR-07-2088 Published June 2008.

[33] Majeed H. M. Antimicrobial Activity of Aqueous, Ethanolic Extracts of Nigella Sativa (Black Cumin) Against Pathogenic Bacteria Isolation from Digestive Tract in Poultry, Indian journal od forensic medidicne & toxicology, 2019, vol.13, issue 4, DOI:10.5958/0973-9130.2019.00360.8

[34] Karameşe, Murat; Özgür, Didem, The antibacterial and antifungal activities of commonly used herbal oils, Journal of Experimental & Clinical Medicine Apr 2020, Vol. 37 Issue 2, p47-51. 5p. http://search.ebscohost.com.

[35] Zeinab K, Mariam S, Seyede E, Arezoo S, Reduced IKK/NF- kB Expression by Nigella Sativa Extract in Breast Cancer, Middle East Journal of Cancer; April 2020; 11(2): 150-158. DOI: http://mejc.sums.ac.ir/article_46414_371c2b4210566dedd0a5ce9b1a7ae07c.pdf

[36] Mohammad Y. A., Investigating the protective effect of Nigella sativa against the cyclophosphamide induced genotoxicity in rats., 2020, Research Journal of Aleppo University, 138: 1-8, Feb. DOI: https://www.researchgate.net/profile/Mohammad_Abajy/publication/340128754 pdf

[37] Kamal K, Dr. Sahni Y.P., Sharma R.K., Vidhi G., Alka S. In vitro antibacterial activity of Panchgavya, Nigella sativa and Asparagus racemosus. The Pharma Innovation Journal 2019; 8(8): 307-310. DOI: http://www.thepharmajournal.com/archives/2019/vol8issue8/PartE/8-8-20-549.pdf

[38] Ahmad A, Habeeb K, Mohd A, Arshad H. Fahd Mohammad, Khaled S. Antibacterial, Antibiofilm and Anticancer Activity of Biologically Synthesized Silver Nanoparticles Using Seed Extract of Nigella sativa., 2020, Basel, Switzerland. DOI.org/10.3390/pr8040388

[39] Ahmad M., Ágnes T., Gabriella S., Silla M., Gabriella K., Antibacterial and Resistance Modifying Activities of Nigella sativaEssential Oil and its Active Compounds Against Listeria monocytogenes., 2018, in vivo 32: 737-743 DOI:10.21873/invivo.11302.

[40] Tímea M., Ahmad M., Munira H., Annamária K, Csilla M., Gabriella K. Gabriella S., Bioactive Compounds of Nigella Sativa Essential Oils Antibacterial Agents against ChlamydiaTrachomatis D, 2019, Microorganisms 2019, 7, 370; DOI:10.3390/microorganisms7090370.

[41] Cut A., Urip H., Muhammad I., The Effect of Black Cumin Oil (Nigella Sativa Oil) Supplementation to Prevent Antiproliferative Action of Temozolamide on Hippocampal Neuronal Stem Cells., Cut Azwanidar / International Journal of Research Publications (IJRP.ORG),2020 Volume-45, Issue-1, January 2020, DOI: 10045112020941.

[42] AysenurA., Fulya G., Lutfiye Y., Buket U. Yasemin Celik A., Mehmet C., Mehmet N., Biogenic platinum nanoparticles using black cumin seed and their potential usage as antimicrobial and anticancer agent., Journal of Pharmaceutical and Biomedical Analysis, 2020, Volume 179, 5 February 2020, 112961., DOI.org/10.1016/j.jpba.2019.112961.

[43] Safiya B. Abdul M., A Review on Nigella sativa: A Marvel Herb, Journal of Drug Delivery and Therapeutics, 2020; 10 (2) :213-219 DOI.org/10.22270/jddt.v10i2.3913.

[44] Yamna K., Basir S., Shabana U. S., Obaid B., Aftab Am, Antiproliferative and apoptotic effects of proteins from black seeds (Nigella sativa) on human breast MCF-7 cancer cell line BMC Complementary Medicine and Therapies volume 20, Article number: 5 (2020)., DOI: 10.1186/s12906-019-2804-1.

[45] Hacer A., Esra Ş, Simge A., Pınar Ş., Determination of Antimicrobial Activity of Different Essential Oils Obtained from Plants on Staphylococcus aureus Strains Isolated from Foods., 2020, vol. 8 no.4 DOI:10.24925/turjaf.v8i4.1012-1017.336

APPENDIX-A

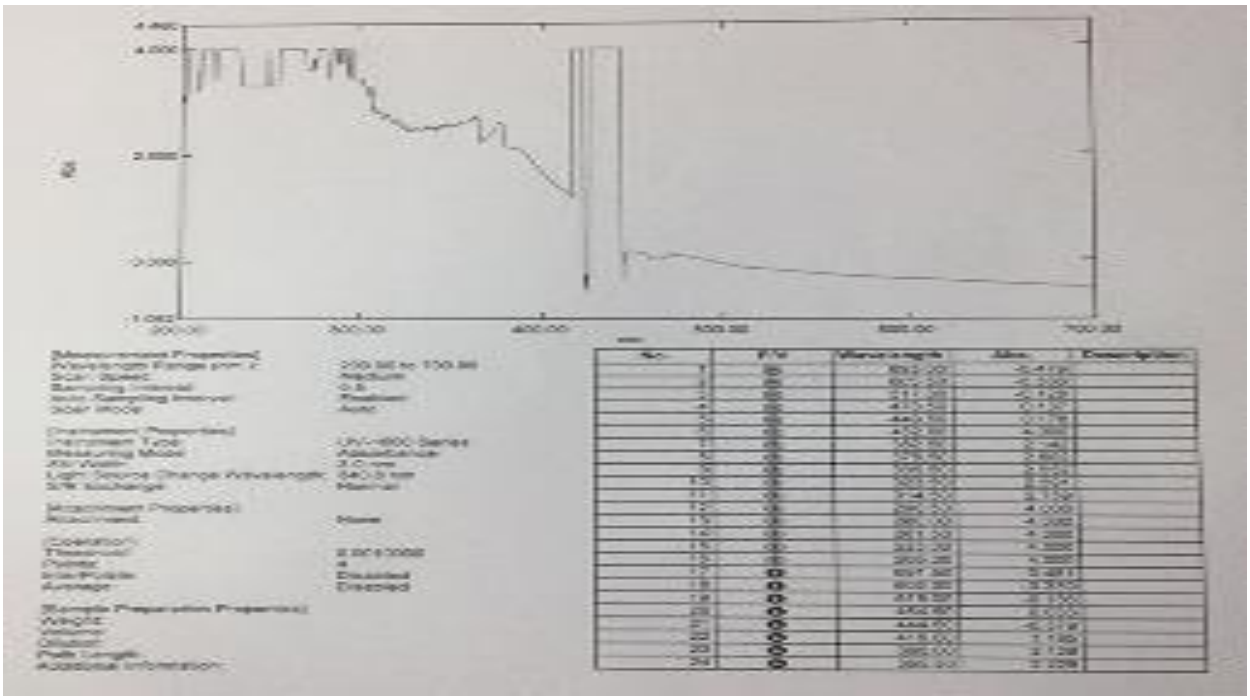

Figure. UV –VIS scanning of *Nigella sativa* extracts with scanning parameters.

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Table. Spectrum data report of extraction solution.

Wave length nm	RawData	RawData	RawData	RawData	RawData	RawData	RawData	RawData
400	0.608	0.376	0.212	0.168	0.150	0.130	0.125	0.125
401	0.607	0.380	0.219	0.177	0.159	0.139	0.134	0.134
402	0.606	0.385	0.227	0.185	0.168	0.145	0.143	0.143
403	0.605	0.389	0.236	0.194	0.178	0.155	0.153	0.153
404	0.606	0.395	0.244	0.203	0.188	0.168	0.163	0.163
405	0.607	0.400	0.252	0.212	0.197	0.177	0.173	0.173
406	0.608	0.405	0.261	0.221	0.206	0.187	0.183	0.183
407	0.609	0.411	0.269	0.230	0.216	0.197	0.193	0.193
408	0.612	0.417	0.278	0.239	0.226	0.207	0.203	0.203
409	0.615	0.424	0.287	0.249	0.236	0.217	0.213	0.213
410	0.618	0.431	0.296	0.258	0.246	0.228	0.224	0.224
411	0.622	0.438	0.306	0.268	0.257	0.238	0.234	0.234
412	0.626	0.445	0.315	0.278	0.267	0.249	0.245	0.245
413	0.629	0.452	0.325	0.288	0.277	0.259	0.255	0.255
414	0.634	0.459	0.334	0.297	0.287	0.270	0.266	0.266
415	0.622	0451	0.326	0.292	0.282	0.264	0.260	0.260
416	0.577	0.408	0.268	0.252	0.243	0.225	0.221	0.221
417	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
418	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
419	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
420	4.000 @	4.000 @	4.000 @	4.000 @	4.000 @	4.000 @	4.000 @	4.000 @
421	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
422	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
423	1.699	1.699	1.699	1.699	1.699	1.699	1.699	1.699
424	-0.602	-0.602	-0.602	-0.602	-0.602	-0.602	-0.602	-0.602
425	-0.602	-0.602	-0.602	-0.602	-0.602	-0.602	-0.602	-0.602
426	1.699	1.699	1.699	1.699	1.699	1.699	1.699	1.699
427	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
428	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
429	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
430	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
431	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
432	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
433	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
434	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
435	4.000 @	4.000 @	4.000 @	4.000 @	4.000 @	4.000 @	4.000 @	4.000 @
436	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
437	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
438	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
439	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
440	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
441	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
442	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
443	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
444	1.699	1.699	1.699	1.699	1.699	1.699	1.699	1.699
445	-0.200	-0.313	-0.391	-0.420	-0.421	-0.436	-0.438	-0.438
446	-0.012	-0.123	-0.200	-0.229	-0.233	-0.245	-0.248	-0.248
447	0.056	-0.052	-0.129	-0.158	-0.159	-0.174	-0.176	-0.176
448	0.068	-0.039	-0.115	-0.143	-0.145	-0.159	-0.161	-0.161
449	0.079	-0.027	-0.101	-0.130	-0.131	-0.145	-0.148	-0.148
450	0.088	-0.17	-0.090	-0.119	-0.120	-0.134	-0.136	-0.136