Application of High-Performance Thin Layer Chromatography and Near Infrared Spectroscopy in the validation of in-use stability of herbal medicines used in the management of Covid-19

RAPHAEL J. SHEDAFA[1]*, JOSEPH SEMPOMBE[1], HAMISI M. MALEBO[2], JOSEPH OTIENO[3], ELIANGIRINGA KAALE[1, 4], MARY TEMU[5] AND PETER IMMING[6]

[1]*Department of Medicinal Chemistry, Muhimbili University of Health and Allied Sciences, Tanzania P.O. Box 65001, Dar es Salaam, Tanzania.*

[2]*UNESCO National Commission of the United Republic of Tanzania, 7 Magogoni Street, P.O. Box 20384, Dar es Salaam, Tanzania.*

[3]*Institute of Traditional Medicine-, Muhimbili University of Health and Allied Sciences, Tanzania P.O. Box 65001, Dar es Salaam, Tanzania.*

[4]*Pharm R&D Laboratory, School of Pharmacy, Muhimbili University of Health and Allied Sciences P.O. Box 65001, Dar es Salaam, Tanzania.*

[5]*Department of Pharmaceutics, Muhimbili University of Health and Allied Sciences, Tanzania P.O. Box 65001, Dar es Salaam, Tanzania.*

[6]*Institut fuer Pharmazie, Martin-Luther-Universitaet Halle-Wittenberg, 06120 Halle, Germany*

Stability testing is a requirement in the registration process for all medicinal products, including herbal medicinal products (HMPs). This study was designed to investigate the stability of two herbal liquid preparations, Covidol® and Rona®, used to treat Covid-19 in Tanzania. The study aimed to predict the effect of standard environmental conditions on the in-use stability of liquid formulations of Covidol® and Rona® stored at ambient conditions for 28 days after opening. The chemical stability patterns of the two products were monitored using high-performance thin layer chromatography (HPTLC) and near infrared spectroscopy (NIRS) methods. Physical parameters such as pH, appearance and odor were also monitored. Under ambient conditions, Covidol® remained stable throughout the study period while Rona® underwent degradation as indicated by changes in its HPTLC and NIRS fingerprint patterns. These methods can be applied for quality control and stability testing of Covidol® and Rona® herbal products.

INTRODUCTION

Natural products of plant origin are distinguished from synthetic drug substances by their structural diversity.[1–4] Currently, many herbal products with various therapeutic effects are commercialized worldwide.[5] Despite the widespread availability and acceptability of herbal preparations in health care systems in many countries, the concern for quality control is emphasized in conventional medicines. Determining shelf life by evaluating the therapeutic efficacy and chemical quality of herbal formulations and products is a challenging task.[6] Herbal formulations usually contain several different compounds, thus making monitoring individual components difficult.[7] The biggest problem facing herbal formulations is the lack of a complete evaluation of their ingredients due to their complex nature.[8,9] During the production process, many changes in chemical structure may occur in the formulation scheme of many products, which could make the product unstable.[10]

* Author to whom correspondence may be addressed. Email address: elia.kaale@gmail.com

In-use stability study is a technique used to establish the stability of a multidose formulation that could affect its quality within accepted specifications as a function of post-opening time. It provides information on the period during which the product can be used safely once the container is opened.[11]

Principally, due to the physical and chemical properties of herbal products, the first opening of a container and seal breaking could pose a risk to the quality attributes such as drug content or microbiological contamination.[12,13] An in-use stability study serves to ensure the continued integrity of product quality after the first opening. This is an important quality parameter that must be performed for a particular herbal formulation to determine its response to exposure to environmental conditions when the container is opened for product consumption.[14]

Near infra-red (NIR) spectroscopy is currently a well-established analytical tool in the field of testing natural medicines [15]. Previous reviews summarize accomplishments in NIR analysis hitherto [16–18]. On the other hand, (HPTLC) analytical instruments are widely used due to their simplicity, small sample requirement, and the ability to analyze multiple samples simultaneously.[19–21]

In recent years, Tanzania has seen tremendous growth in herbal drugs used for Covid-19 management.[22–24] Consequently, several proprietary herbal formulations, mainly polyherbals have been developed by domestic manufacturers.[25] Recent advances in photochemistry have demonstrated that many of these herbal constituents may react with each other, thus raising concerns about the stability of the formulations [26].

Covidol® is registered by the Traditional and Alternative Medicine Council as an oral liquid, exclusively composed of medicinal plant extracts as active ingredients, namely: *Acalypha fruticosa, Fracourtia indica, Keetia venosa, Warburgia ugandensis, Ximenia caffra, and Zanthoxylum chalybeum.* This herbal remedy is promoted for management of Coronavirus Disease-19 (Covid-19), seasonal flu, and respiratory disorders. Similarly, Rona® is a herbal formulation containing *Glycyrrhiza, Moringa, Artemisia,* and *Zingiber extract* and is used for muscle aches, sore throat, coughing, and chest tightness.

MATERIALS AND METHODS

Chemicals and reagents

Analytical-grade methanol, formic acid and toluene were purchased from Loba Chemie PVT Ltd (Mumbai, India), while acetone was obtained from Carlo Erba (Val-de-Reuil, France).

Samples collection

Eighty-eight (88) samples consisting of 56 Covidol® and 32 Rona® items were purchased from various private pharmacies in Dar es Salaam. Product information such as manufacturer name, manufacturing date, and expiration date were documented at the time of purchase.

Preparation of test solutions

The test solution was prepared by diluting 25 ml of the sample to 50 ml using methanol-water (50:50 v/v). The resulting mixture was sonicated for five minutes and filtered. Five µl of the clear supernatant was applied to pre-coated HPTLC silica gel 60 F_{254} plates of dimensions 20 cm × 10 cm.

HPTLC analysis

All samples were analyzed on a CAMAG HPTLC system equipped with a sample applicator and digital visualizer (CAMAG, Muttenz, Switzerland), a CAMAG TLC 4 scanner, and Linomat V for Vision Cats version 2.5 data acquisition software. HPTLC silica gel 60 (F254) was used as the stationary phase and detection were performed under ultraviolet light at 254 nm and 366 nm. The mobile phase consisted of toluene - acetone - formic acid (45:45:10 v/v), while HPTLC densitogram images were converted to datasets using the Vision Cats software.

Physical evaluation

Each stability sample was evaluated for colour, pH, and odor. For pH determination, 50 ml of solution was poured into a beaker and measurement made on a pH meter suitably calibrated with buffer solutions of pH 4.0, 7.0 and 9.0.

NIR spectra collection

NIR measurements were performed using a LabSpec-5000 spectrophotometer (ASD Inc, Alpharetta, GA, USA) equipped with Indico Pro software 3.0. Each sample was scanned at a wavelength range of 350 - 2500 nm. Spectra of liquid samples were run using standard sample holders and one ml cuvette.

NIR model development and validation

A training set was developed using five samples of each batch of Covidol® and Rona® products. Each sample was divided into ten portions and scanned three times thus yielding a total of 150 spectra for each batch in Analytical Spectral Devices (ASD) format (Table 1).

The spectra obtained were converted into a training file using Grams/AI™ chemometric software (Thermo Fisher Scientific, Waltham, MA, USA). All spectra were recorded as log (1/R) and were converted into SPC format for data processing.

Three samples each of Covidol® and Rona® were used as predictors for the validation of the training model. Each sample was divided into ten portions and scanned in triplicate, giving a total of 90 scans of each batch.

In-use stability study by NIR spectroscopy

The in-use-stability of Covidol® was monitored by comparing the change in spectra weekly for 28 days. The parameter used was the Mahalanobis distance (M-Distance), where the acceptance criteria were 1 for 'accept' and 3 for 'reject'. The Mahalanobis distance is used as a screening technique for consistency, to demonstrate whether some aspect of the sample differs from the reference spectra used to build the method.

Table 1. Number of herbal product samples used in the study.

Product name	Batch No.	Training set samples	Validation set samples
Covidol®	343	5	3
	350	5	3
	351	5	3
	352	5	3
	353	5	3
	367	5	3
	381	5	3
Rona®	01	5	3
	02	5	3
	03	5	3
	04	5	3

Data analysis

Retardation factor (Rf) values of the bands on the images and densitometric analysis was carried out using CAMAG TLC Scanner 4, fitted with vision Cat planar chromatography manager software (CAMAG, Muttenz, Switzerland, version 2.5).[27] The Gram IQ software (Thermo Fisher Scientific, Waltham, MA, USA) was employed in the determination of Mahalanobis distances from NIR spectroscopy.

RESULTS AND DISCUSSION

Physical Evaluation

Both Covidol® and Rona® were presented as brownish liquids. Visual inspection showed no appreciable changes in the colour or appearance of the samples during the 28 days of the study. Sample pH ranged from 6.7 - 6.5 for Covidol® and 5.8 - 5.2 for the Rona® (Table 2). The change in pH was less than 5% from its original value and was therefore acceptable.[28,29]

Table 2. Physical properties of the herbal products under study

Test parameter	Days	Covidol®	Rona®
pH	0	6.7 ± 0.01	5.8 ± 0.01
	7	6.6 ± 0.01	5.5 ± 0.01
	14	6.6 ± 0.01	5.4 ± 0.01
	28	6.5 ± 0.01	5.2 ± 0.01
Odour	0	Characteristic	Characteristic
	7	Characteristic	Characteristic
	14	Characteristic	Characteristic
	28	Characteristic	Characteristic
Colour	0	Brownish	Brownish
	7	Brownish	Brownish
	14	Brownish	Brownish
	28	Brownish	Brownish

pH-Values are expressed as mean $\pm$ SD (n =3)

The pH value is one of the major factors affecting the quality of a drug with respect to chemical and microbial degradation. Various researchers found that low (acidic) pH reduces bacterial contamination, whereas, neutral or alkaline pH , causes higher contamination in herbal medicines.[30]

In-use stability study using HPTLC.

Covidol® samples remained relatively stable during the study period as demonstrated by consistency of the HPTLC profile (Figure 1) and densitograms (Figure 2). In contrast, Rona® showed degradation that was exhibited by the decrease in the number of peaks at Rf 0.5 to 0.9 on day 28 (Figure 3 & Figure 4) compared to the same peaks at 0 - 14 days. On a similar day interval, the HPTLC profile for Covidol® (Figure 1 & Figure 2) remained unchanged.

The HPTLC profile of Rona®, at 366 nm (Figure 3), showed a decrease in bands on day 28 compared to day 0, which reflects sample degradation. The results are consistent with the NIR results (Figure 6).
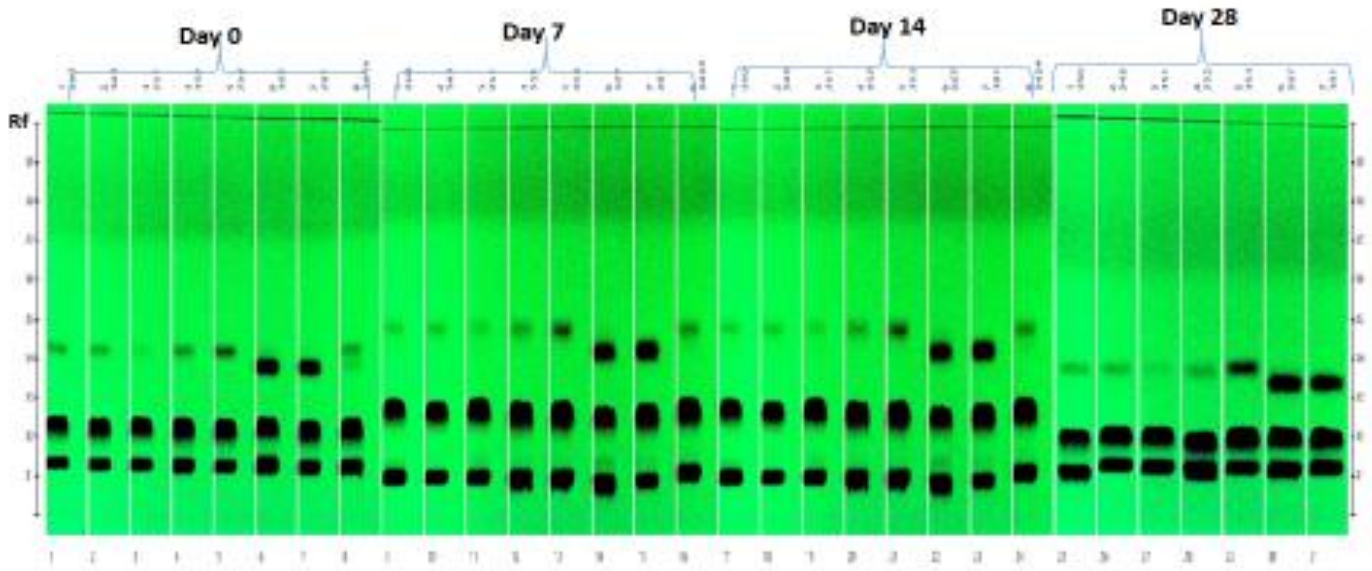
Figure 1:   HPTLC fingerprinting of Covidol® at UV 254 nm from 0 to 28 days.
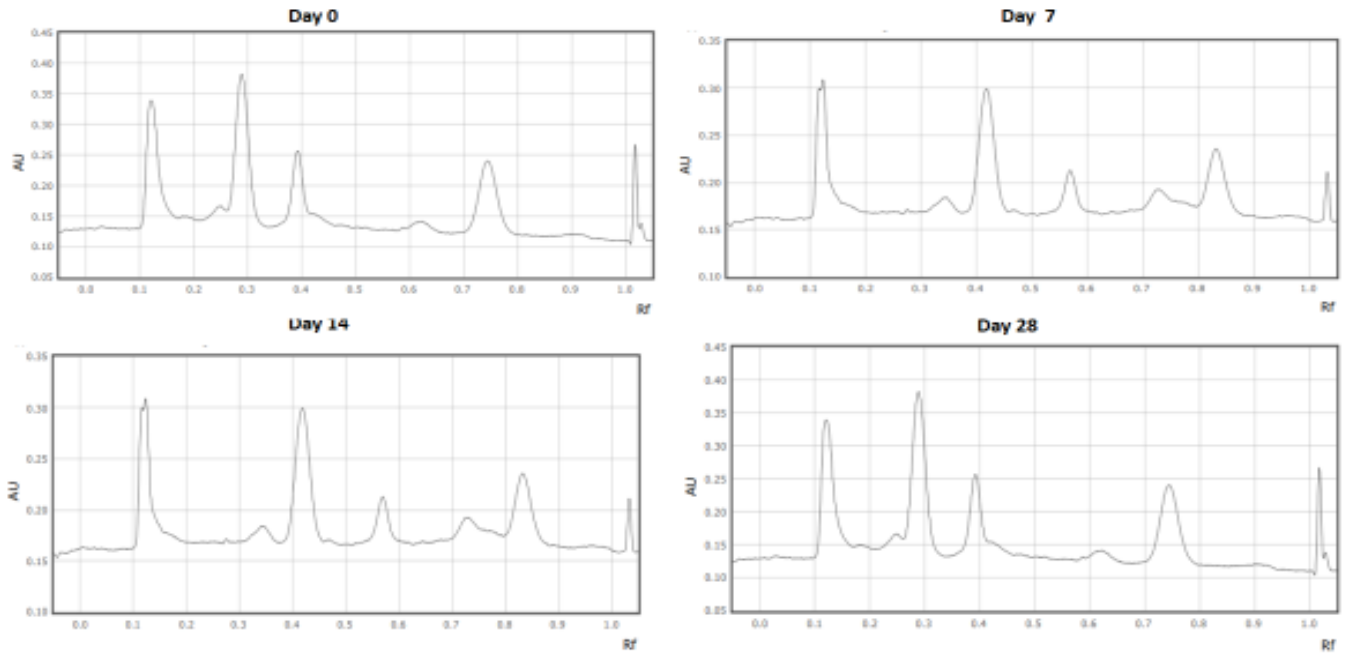

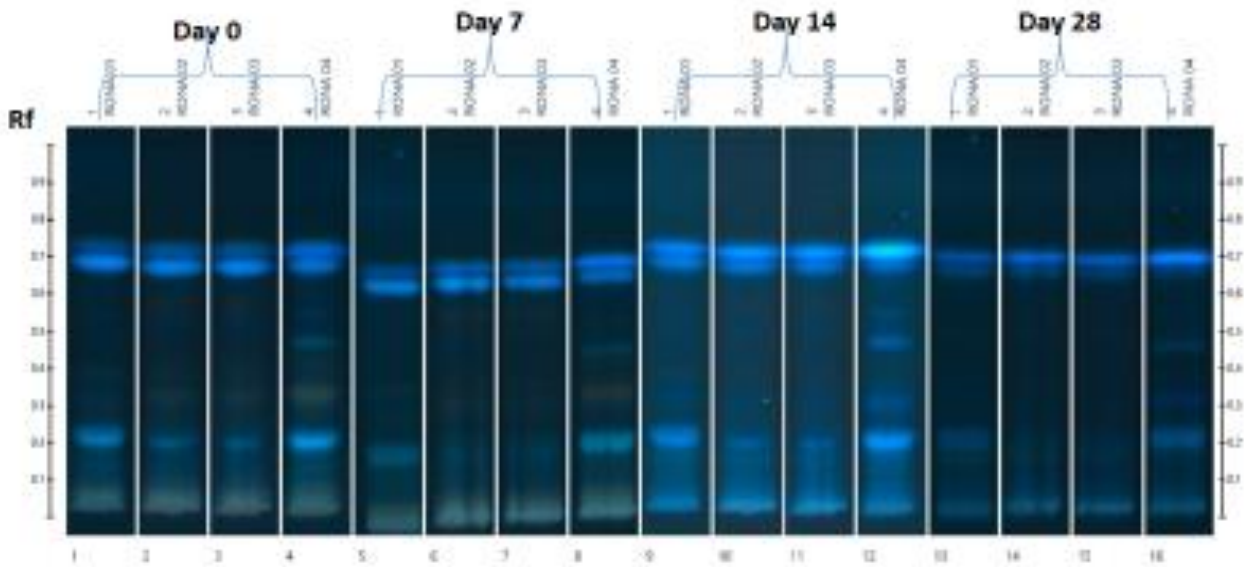**Figure 2:   HPTLC densitograms for Covidol® at UV 254 nm.**

Figure 3:   HPTLC fingerprinting of Rona® at UV 366 nm from 0 to 28 days.
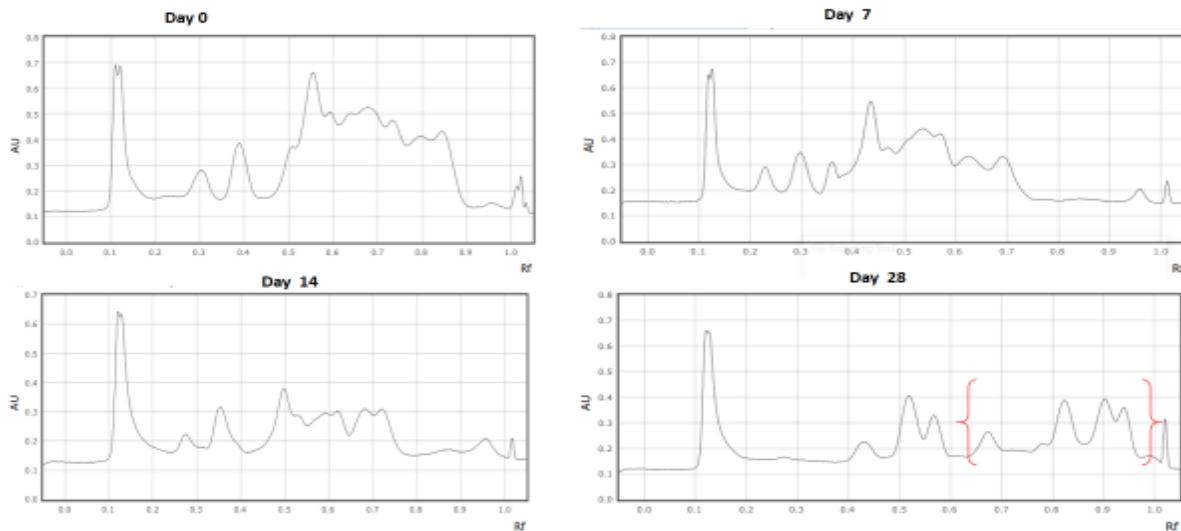

Figure 4:   HPTLC densitograms for Rona® at UV 366 nm.

In-use stability study by NIR spectroscopy

During the NIR analysis, the training set served as a reference, whereas the validation set was used to confirm the classification results based on the analysis used by the actual batch. The NIR reflection spectra shown in Figure 5 represent comparisons of overlaid spectra of Covidol® from days 0 to 28. No significant changes were observed in the spectral regions recorded in the product within 28 days of the study. The NIR spectra shown in Figure 6 showed that the M-Distance for day 28 was more than 3, indicating that there were chemical interactions within the product as shown by the change in the spectra region from 1500 nm to 2100 nm.
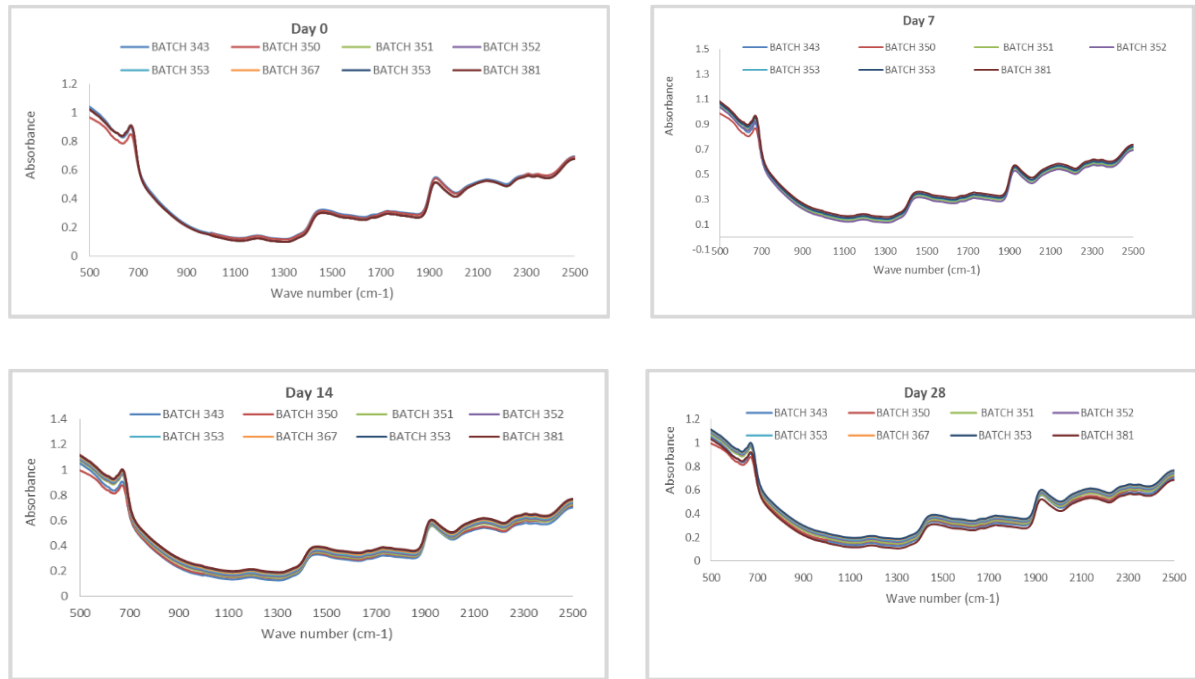
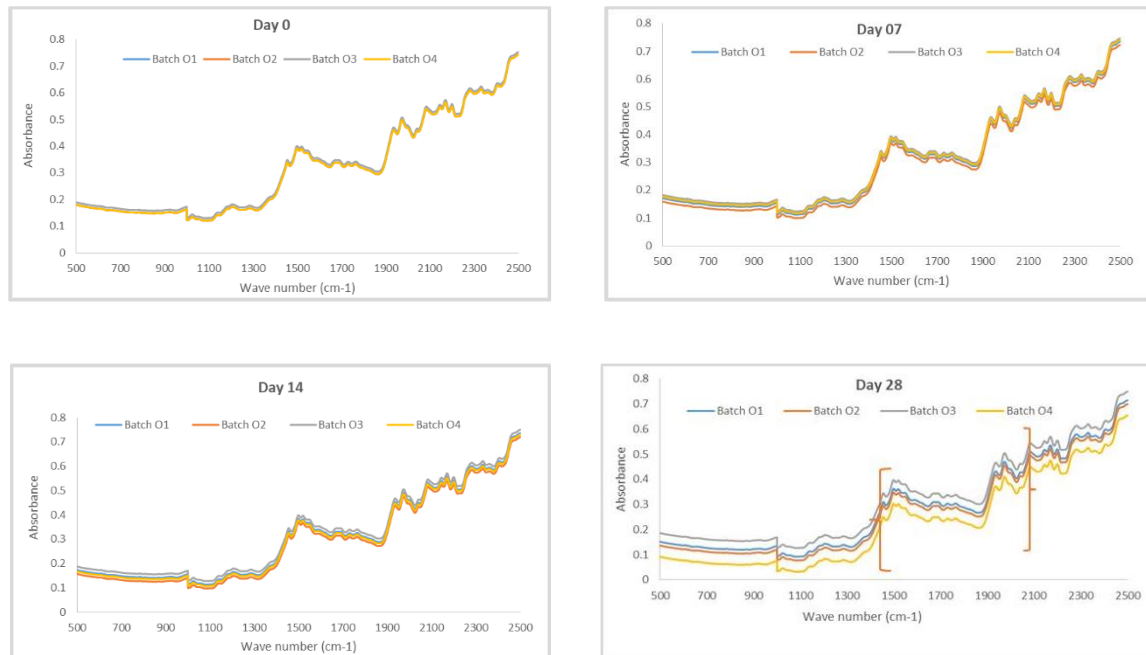**Figure 5: NIR spectra comparisons for Covidol® from 0 to 28 days.**

Figure 6:   NIR spectra comparisons for Rona® from 0 to 28 days.

Storage factors such as temperature, light, and oxygen have been shown to affect product stability.[31,32] The container opening approach used in this study was designed to mimic the routine use of the medicine. Furthermore, many users were likely to keep the medicine longer than the specified time of seven days due to the high cost. Results of previously studies have demonstrated a profound effect of the storage temperature on the stability of the products.[33] Current research shows that oxidation reactions may occur, thus resulting in changes in the chemical profile of the Rona®, while exposure to light may further accelerate its decomposition.[34]

CONCLUSION

This study highlights the importance of proper storage of herbal liquid formulations from temperature and humidity effects in order to maintain the physical integrity and stability of the product during the stated shelf life. Two liquid herbal formulations, Covidol® and Rona®, were evaluated whereby Covidol® remained physico-chemically stable when stored at ambient temperature for 28 days compared to Rona® which degraded under the same conditions. These results are important because, during the Covid-19 pandemic, it was common for patients to extend the use of herbal products beyond the specified period (seven days) after opening, oblivious of the risks posed by chemical and microbial degradation. This study underscores the need for patients to adhere to proper storage conditions of medicine during use. The HPTLC and NIR spectroscopy methods proposed in this study, permit tracking of variations in phytochemical fingerprint patterns and thus could be employed in detecting deterioration in the quality of herbal formulations.

REFERENCES

(1)   Bodeker, G. WHO Global Atlas on Traditional, Complementary and Alternative Medicine (TCAM). *Iran. J. Pharm. Res.* **2010**.

(2)   Jacobson, J. S. Traditional, Complementary and Alternative Medicine: Policy and Public Health Perspectives. *Glob. Public Health* **2010**, 5 (2), 206-208.

(3)   Chen, D. F.; Wu, Y. F.; Li, H. Y.; Wang, M. C.; Zhou, Y. Z.; Zhu, Z. F.; Liu, Y. Z.; Pan, X.; Zhou, J.; He, F. Y. Visual Atlas A nalysis on Documents of Traditional Chinese and Western Medicine for Treatment of Spastic Cerebral Palsy Based on CiteSpace Bibliometrics. *Chinese Tradit. Herb. Drugs* **2021,** 52 (14), 4318-4326.

(4)   Lim, D. Pocket Atlas of Chinese Medicine. *Focus Altern. Complement. Ther.* **2010**, 14 (4), 353-353.

(5)   Sofowora, A.; Ogunbodede, E.; Onayade, A. The Role and Place of Medicinal Plants in the Strategies for Disease Prevention. *African journal of traditional, complementary, and alternative medicines : AJTCAM / African Networks on Ethnomedicines*. **2013,** 10 (5), 210-229.

(6)   Bansal, G.; Suthar, N.; Kaur, J.; Jain, A. Stability Testing of Herbal Drugs: Challenges, Regulatory Compliance and Perspectives. *Phytotherapy Research.* **2016,** 30 (7), 1046-1058.

(7)   Ekor, M. The Growing Use of Herbal

Medicines: Issues Relating to Adverse Reactions and Challenges in Monitoring Safety. *Frontiers in Neurology*. **2014**.

(8) Mukherjee, P. K. Quality Evaluation of Herbal Medicines: Challenges and Opportunities. In *Quality Control and Evaluation of Herbal Drugs*; 2019.

(9) Mirzaeian, R.; Sadoughi, F.; Tahmasebian, S.; Mojahedi, M. The Role of Herbal Medicines in Health Care Quality and the Related Challenges. *J. HerbMed Pharmacol.* **2021;** pp 53-57.

(10) Kruse, S. O.; Sultan, K. Stability Testing of Herbal Medicinal Products. *Innovations in Pharmaceutical Technology*. **2010.**

(11) Chavda, H. In-Use Stability Studies: Guidelines and Challenges. *Drug Development and Industrial Pharmacy*. **2021.**

(12) de Sousa Lima, C. M.; Fujishima, M. A. T.; de Paula Lima, B.; Mastroianni, P. C.; de Sousa, F. F. O.; da Silva, J. O. Microbial Contamination in Herbal Medicines: A Serious Health Hazard to Elderly Consumers. *BMC Complement. Med. Ther.* **2020**.

(13) Ndhlala, A. R.; Ncube, B.; Van Staden, J. Ensuring Quality in Herbal Medicines: Toxic Phthalates in Plastic-Packaged Commercial Herbal Products. *South African Journal of Botany*. **2012,** pp 60-66.

(14) Thakur, L.; Ghodasra, U.; Patel, N.; Dabhi, M. Novel Approaches for Stability Improvement in Natural Medicines. *Pharmacognosy Reviews*. **2011,**5 (9),48-54.

(15) Beć, K. B.; Grabska, J.; Huck, C. W. NIR Spectroscopy of Natural Medicines Supported by Novel Instrumentation and Methods for Data Analysis and Interpretation. *Journal of Pharmaceutical and Biomedical Analysis*. **2021**.

(16) Huck, C. Infrared Spectroscopic Technologies for the Quality Control of Herbal Medicines. In *Evidence-Based Validation of Herbal Medicine*; **2015**.

(17) Huck, C. W. Miniaturized MIR and NIR Sensors for Medicinal Plant Quality Control. *Spectrosc. (Santa Monica)* **2017**, pp 477-493.

(18) Wei, X. C.; Cao, B.; Luo, C. H.; Huang, H. Z.; Tan, P.; Xu, X. R.; Xu, R. C.; Yang, M.; Zhang, Y.; Han, L.; Zhang, D. K. Recent Advances of Novel Technologies for Quality Consistency Assessment of Natural Herbal Medicines and Preparations. *Chinese Medicine (United Kingdom)*. **2020.**

(19) Kaur, I.; Suthar, N.; Kaur, J.; Bansal, Y.; Bansal, G. Accelerated Stability Studies on Dried Extracts of Centella Asiatica Through Chemical, HPLC, HPTLC, and Biological Activity Analyses. *J. Evidence-Based Complement. Altern. Med.* **2016,** 21 (4), 127-137.

(20) Frommenwiler, D. A.; Booker, A.; Vila, R.; Heinrich, M.; Reich, E.; Cañigueral, S. Comprehensive HPTLC Fingerprinting as a Tool for a Simplified Analysis of Purity of Ginkgo Products. *J. Ethnopharmacol.* **2019**.

(21) Frommenwiler, D. A.; Kim, J.; Yook, C. S.; Tran, T. T. T.; Cañigueral, S.; Reich, E. Comprehensive HPTLC Fingerprinting for Quality Control of an Herbal Drug – The Case of Angelica Gigas Root. *Planta Med.* **2018**.

(22) Liwa, A.; Roediger, R.; Jaka, H.; Bougaila, A.; Smart, L.; Langwick, S.; Peck, R. Herbal and Alternative Medicine Use in Tanzanian Adults Admitted with Hypertension-Related Diseases : A Mixed-Methods Study. *Int. J. Hypertens.* **2017**, *2017*.

(23) Ejike, C. E. Covid-19 and African Traditional Medicines. *J. African Stud. Sustain. Dev.* **2020**,3 (11), 1-13.

(24) Richey, L. A.; Gissel, L. E.; Kweka, O. L.; Bærendtsen, P.; Kragelund, P.; Hambati,

H. Q.; Mwamfupe, A. South-South Humanitarianism: The Case of Covid-Organics in Tanzania. *World Dev.* **2021**.

(25)   Mshana, G.; Mchome, Z.; Aloyce, D.; Peter, E.; Kapiga, S.; Stöckl, H. Contested or Complementary Healing Paradigms? Women's Narratives of COVID-19 Remedies in Mwanza, Tanzania. *J. Ethnobiol. Ethnomed.* **2021,** 17 (30), 1-12.

(26)   Chaudhary, R.; Kumari, P. Stability Aspects of Herbal Formulation. WJPLS, **2022**, *8* (2), 103–110.

(27)   Luyangi, F.; Kaale, E.; Manyanga, V.; Mugoyela, V. Development of a Qualitative near Infrared Screening Method for Quality Consistency Assessment of Virgin Sunflower Oil. *Spectr. Anal. Rev.* **2017**,05 (03), 25-36.

(28)   Kim, J. H.; Lee, K.; Jerng, U. M.; Choi, G. Global Comparison of Stability Testing Parameters and Testing Methods for Finished Herbal Products. *Evidence-based Complementary and Alternative Medicine*. **2019**.

(29)   Chair, M. S.; Barnes, A.; Goddard, W.; Hiom, S.; Oldcorne, M.; Precious, N.; Aiyalu, S.; Weir, P. A standard protocol for deriving and assessment of stability Part 3-Oral Liquid Medicines (Solutions, Emulsions, Suspensions and Syrups). *NHS Pharm. Qual. Assur. Comm.* **2014**.

(30)   Rani, S.; Rahman, K.; Younis, P. Accelerated Stability Studies of Sufoofe Sailan: A Unani Formulation. *AYU (An Int. Q. J. Res. Ayurveda)* **2015,** 36, 83.

(31)   de Albuquerque Ugoline, B. C.; de Souza, J.; Caldeira, T. G.; Saúde-Guimarães, D. A. Stability and Degradation Kinetics of an Ethanolic Extract and Tincture of Aerial Parts from Lychnophora Passerina (Brazilian Arnica). *Rev. Bras. Farmacogn.* **2021,** 31 (4),453-462.

(32)   Huynh-Ba, K. *Handbook of Stability Testing in Pharmaceutical Development: Regulations, Methodologies, and Best Practices*; **2009,** pp 1-389.

(33)   Ali, A.; Chong, C. H.; Mah, S. H.; Abdullah, L. C.; Choong, T. S. Y.; Chua, B. L. Impact of Storage Conditions on the Stability of Predominant Phenolic Constituents and Antioxidant Activity of Dried Piper Betle Extracts. *Molecules* **2018,** 23, 484.

(34)   Cortés-Rojas, D. F.; Souza, C. R. F.; Oliveira, W. P. Assessment of Stability of a Spray Dried Extract from the Medicinal Plant Bidens Pilosa L. *J. King Saud Univ. - Eng. Sci.* **2016,**28 (2) ,145-146.