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Target Oriented Synthesis and Mass Spectral Characterization of Curcumin-Phenformin Adduct: Potential Insights into the Role of this Conjugate as Anti-Diabetic and Anti-Cancer Agent

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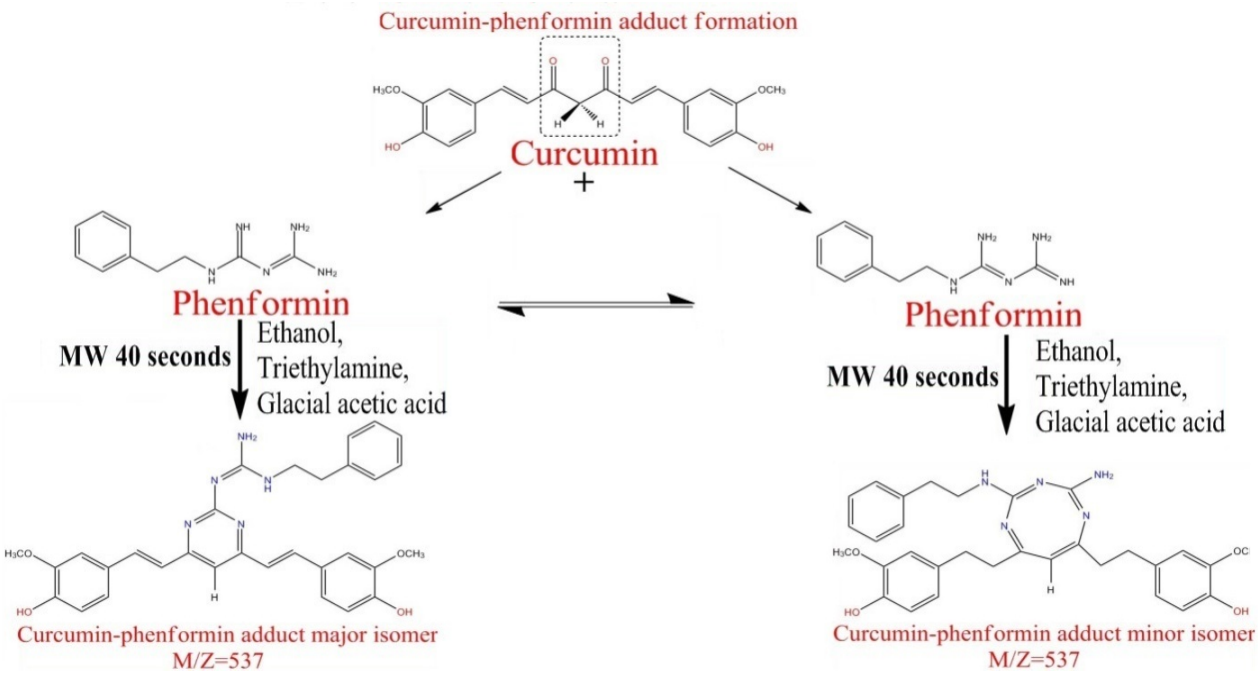
Abstract: Recently microwave-induced chemical synthesis of curcumin-metformin adduct to enhance the efficacy of metformin in preventing the formation ofAdvanced Glycation End Products (AGEs) has been reported from authors' laboratory. The present studies describemicrowave-induced chemical synthesis and mass spectral characterization of curcumin-phenformin adducts using LC-MS/MS. The mechanism of formation and its analytical data via Thin-Layer Chromatography (TLC) combined with MS/MS fragmentation revealed a major six membered ring adduct and a minor eight membered ring isomer. A facile chemical synthesis and identification of major and minor isomers presented in this study may offer novel therapeutic strategies for inhibiting AGEs as well as anti-cancer treatments.

Key words: Diabetes mellitus, advanced glycation end- products, glycated human serum albumin, curcumin, phenformin, metformin, anti-cancer agents.



Graphical abstract: Mechanism of formation of curcumin-phenformin adducts.

1. Introduction

Metformin, phenformin and aminoguanidine are all nucleophilic hydrazino compounds which block the formation of fluorescent AGEs [1-6]. They also prevent glucose-derived collagen cross linked Amadori products *in-vivo* and *in-vitro* [6-9]. Recently microwave-induced chemical synthesis of curcumin-metformin adducts and its characterization via TLC-Electrospray ionization mass spectrometry has been described [10]. Also the combination of metformin with the phytochemicals present in okra seed extract to enhance the efficacy for inhibiting AGEs and their comparison with different AGEs inhibitors has been reported [10-14]. A protective role of metformin, 2-aminoguanidine and phenformin in the impairment of the anti-oxidant properties of human serum albumin in patients with diabetes has been established [1, 5, 7-9]. The anti-cancer and anti-diabetic effects of phenformin and metformin have prompted researchers to investigate their use in clinical medicine [3, 4, 15-26]. Although phenformin is 50 times more potent than metformin as an anti-diabetic drug, it is associated with lactic acidosis side effects [3]. A recent study elucidated that phenformin has better anti-cancer activity than metformin as accompanied by significant inhibition of tumor growth [3].

The exact mechanisms by which metformin and phenformin reduce cancer risk have not been established [19-28]. But a synergistic anti-cancer effect of phenformin combined with sodium oxamate, a known lactate dehydrogenase (LDH) inhibitor reduced the side-effect of lactic acidosis [3, 4]. Inhibition of complex I is cited as the plausible mechanism in these studies [3, 4, 10].

Curcumin (Fig. 1) is a major dietary phenol present in yellow spice turmeric as well as in the plant *Curcuma Long Linn* [29-32]. Its biological activities including antidiabetic and anticancer effects have been extensively studied [10, 32-34]. Curcumin has shown its natural brain protecting action by blocking aggregation and fibril formationin patients from Alzheimer's disease [35-40]. Therefore, in order to enhance biological activity of phenformin and reduce the side effects associated with this medication, the present studies describe a facile microwave-induced chemical synthesis and mass spectral characterization of curcumin-phenformin adduct. Furthermore, understanding the chemical, biochemical and mechanistic implications of this new target molecule may have the potential to serve as more potent anti-diabetic and anti-cancer drugs.

Fig. 1 Curcuma Longa Linn.

2. Materials and Methods

Phenformin (Phenethyl biguanide) was purchased from Sigma Chemicals. Curcumin powder was obtained from Walgreen. Glacial acetic acid was obtained from Sigma-Aldrich. Aluminium backed plates for TLC were obtained from Macherey-Nagel, Germany. Silica Gel (63-200 μm particle size) was purchased from Sigma-Aldrich. Electrospray-Atmospheric pressure Ionization Mass Spectra (ES-API) in the (+*ve*) and (-*ve*) ion mode was recorded using Agilent electrospray mass spectrometer. UV-Visible studies were performed using a Nano drop1000 Spectrophotometer as described previously [31, 34].

2.1 Experimental Procedure

Curcumin (6 mg; 0.012 mM) was dissolved in ethanol (3 mL) and vortexed for 30 seconds followed by heating in the microwave oven for 35 seconds with 3 second intervals. Mixture was vortexed again for

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another 30 seconds and centrifuged at 6,000 RPM (5 mins). The clear supernatant was transferred in a clean vial followed by addition of phenformin hydrochloride (10 mg), triethylamine (300 µL) and a catalytic amount of glacial acetic acid (200 µL). The reaction mixture vortexed and subsequently heated in the microwave oven for 40 seconds at 3 seconds intervals.

The final reaction product was monitored via Thin-layer chromatography (aluminum backed pre-coated SILG/UV254) TLC plates in the solvent system: $CHCl_3$:CH_3OH = 24:4 (v/v) (Fig. 2). The desired compound (10 mg) was evaporated to dryness under N_2 atmosphere at room temperature and the residue chromatographed on preparatory TLC Silica Gel plates (Fig. 3). For purification by Flash Column Silica Gel Chromatography the reaction mixture was applied to a glass column filled with (2.1 g) of silica gel particle size (63-200 µm) as described previously [10]. Elution was performed using 100 mL of a mixture of hexane/ethyl acetate (30:70 v/v) and subsequent elution with increasing amounts of ethyl acetate with hexane (50:50 v/v) provided pure fractions 3,4,9-13 (4.5 mg). UV-Visible Nano drop Spectrophotometry of the staring material Curcumin (Fig. 3) and the curcumin-phenformin adduct is shown in Fig. 4.

Mass spectral characterization was accomplished using LC-MS/MS. Electrospray-Atmospheric Pressure

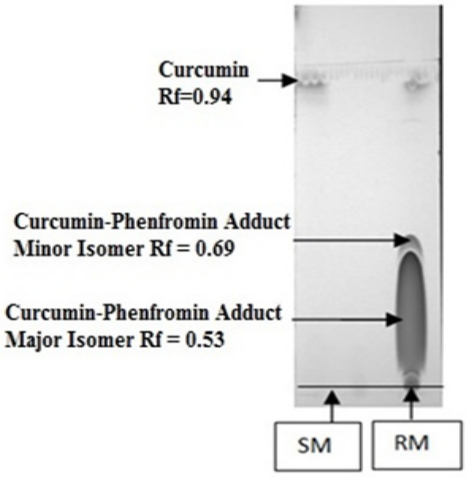

Fig. 2 TLC: $CHCl_3$: CH_3OH = 24:4 (v/v), Curcumin: starting material (SM), reaction mixture (RM).

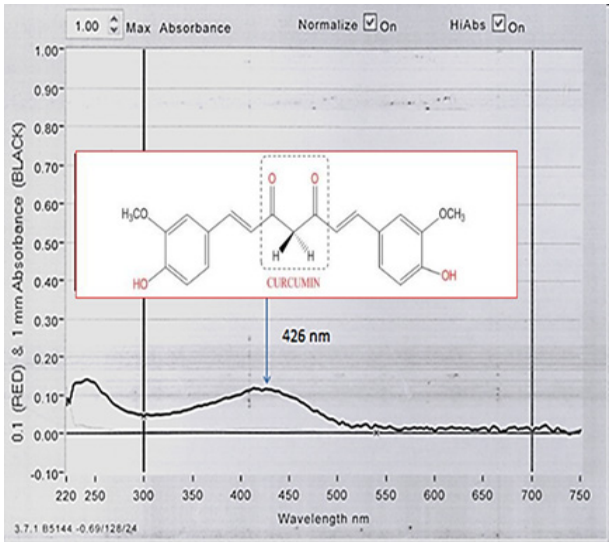

Fig. 3 UV-Vis Nano drop spectrophotometryanalysis of Curcumin.

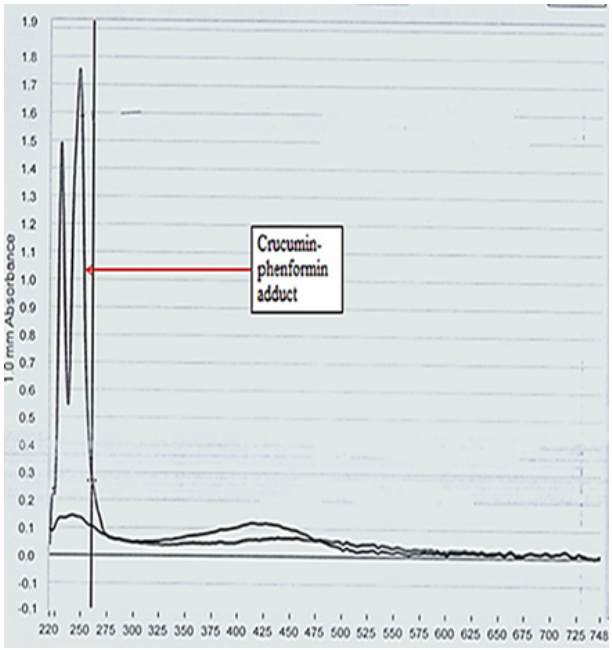

Fig. 4 UV-Vis Nano drop spectrophotometry analysis of Curcumin-phenforminadduct .

Ionization Mass Spectra (ES-API) in the (*+ve*) and (*-ve*) ion mode was recorded using Agilent electrospray mass spectrometer. The mass spectral characterization of the curcumin-phenformin adduct exhibited a molecular ion peak at M/Z = 537 (30% intensity) and a fragment peak at M/Z = 310/311 [M^+−(149+77)] (Fig. 5). The eight memberedring isomer exhibited a mass spectral fragment at M/Z = 354 [M+ - (184), 100% intensity, derived from the fragment (239-55)]

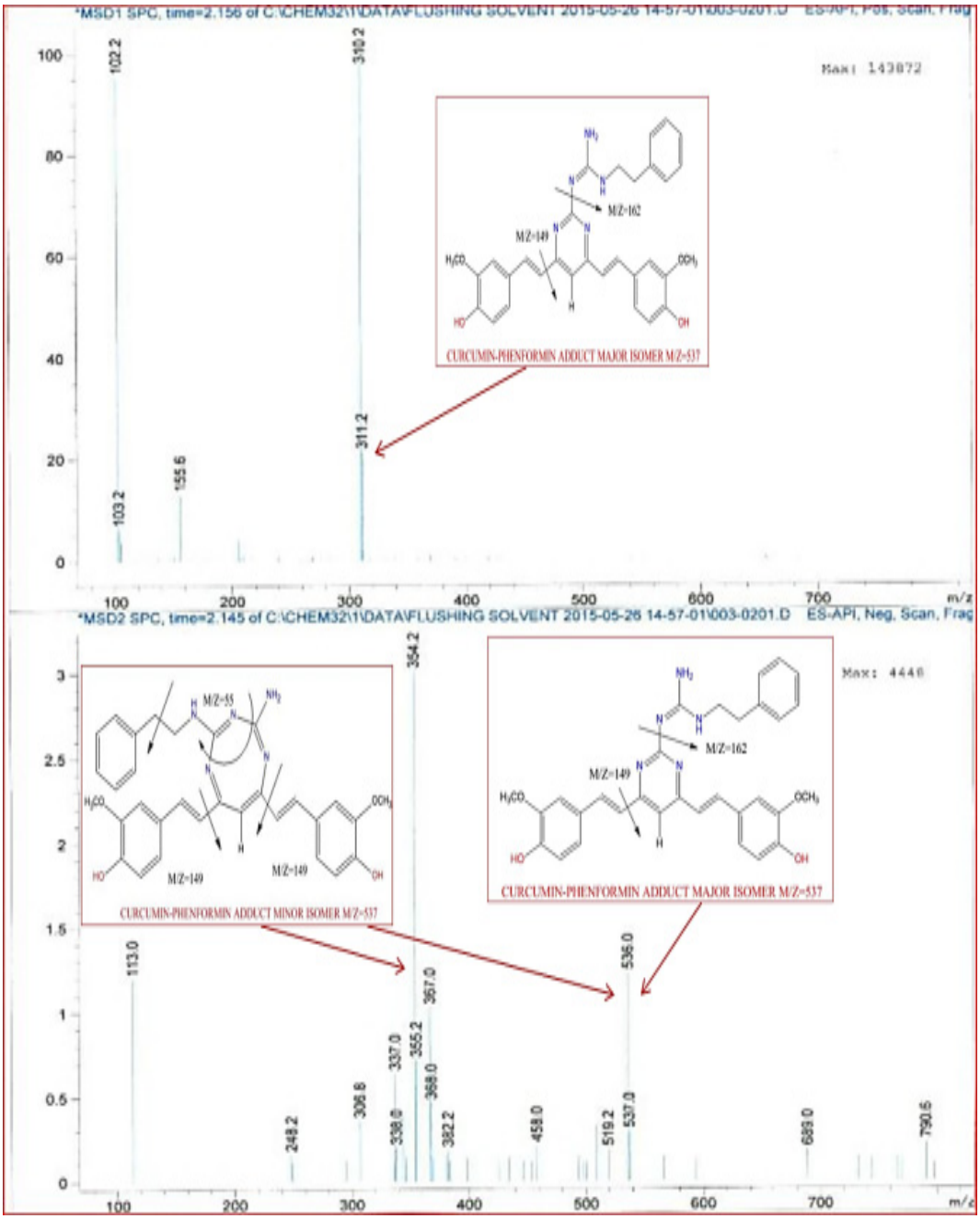

Fig. 5 MS/MS of Curcumin-phenformin major (right panel) and minor (left panel) isomers (M/Z = 537). Major isomer (six membered ring, top right panel) fragment M/Z = 310/311. Minor isomer (eight membered ring, bottom left panel) fragment M/Z = 354.

(Fig. 5). The LC-MS/MS spectrum of the reference standard phenformin M/Z=206/207 recorded in the positive ion mode is shown in Fig. 6.

3. Results and Discussion

Authors recently described comparative efficacies of metformin, phenformin, yellow curcumin and white curcumin in inhibiting the advanced glycation end products AGEs [10-12] (Fig. 7). The efficacies of metformin with OSE and phenformin with yellow curcumin in inhibiting AGEs were 78% and 45% respectively. In a previous publication authors elucidated the reaction mechanism of formation of curcumin-metformin adduct [10].

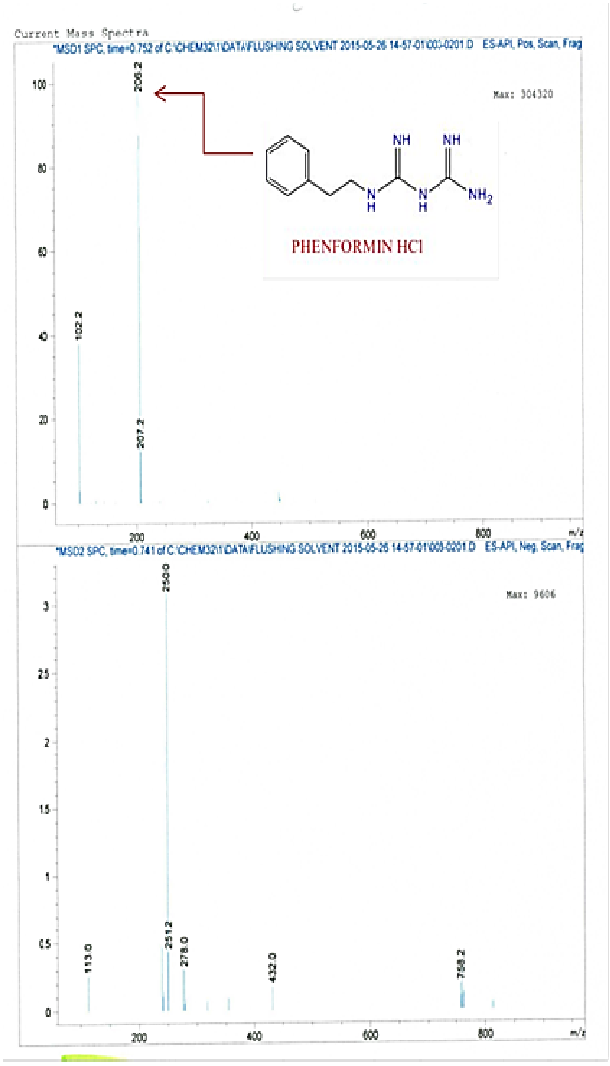

Fig. 6 MS/MS of phenformin (positive ion mode M/Z = 206 starting material).

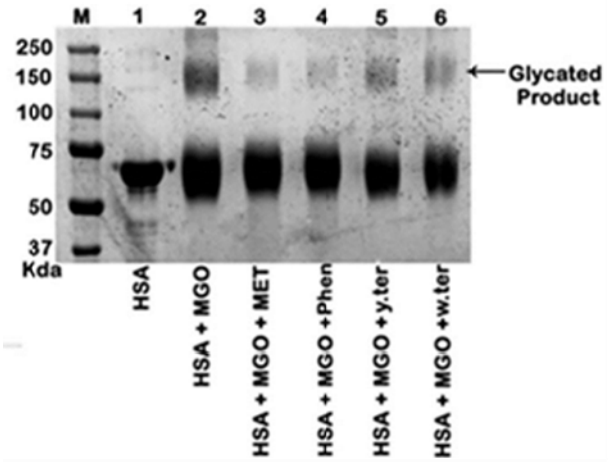

Fig. 7 Comparison of the anti-glycosylation activity of metformin, phenformin, yellow curcumin and white curcumin as shown by SDS-Gels.

Present studies describe a facile synthetic strategy for the formation of major and minor curcumin-phenformin adducts. Target oriented synthesis showed two isomeric forms of adduct in 90% and 10% yield respectively. Mass spectral characterization showed a molecular ion at M/Z = 537 (30% intensity) and a fragmented peak at M/Z =310/311 (100% intensity; *+ve* ion mode) confirming the formation of six membered right isomer as shown (Fig. 5, top right panel). Another major fragment MS/MS in the (*-ve* ion) mode exhibited M/Z = 354 (100% intensity) as shown in Fig. 5 which has been tentatively assigned to the eight membered ring isomer.

Two possible curcumin-phenformin reaction pathways are illustrated (Figs. 8, 9). The highly nucleophilic nature of phenformin exists in two isomeric forms. When reacted with curcumin, its aplanar molecule results are in formation of six and eight membered ring adducts. The authors will isolate in large amounts and investigate the chemistry, biochemistry and mechanistic implications of these two adducts. Authors believe

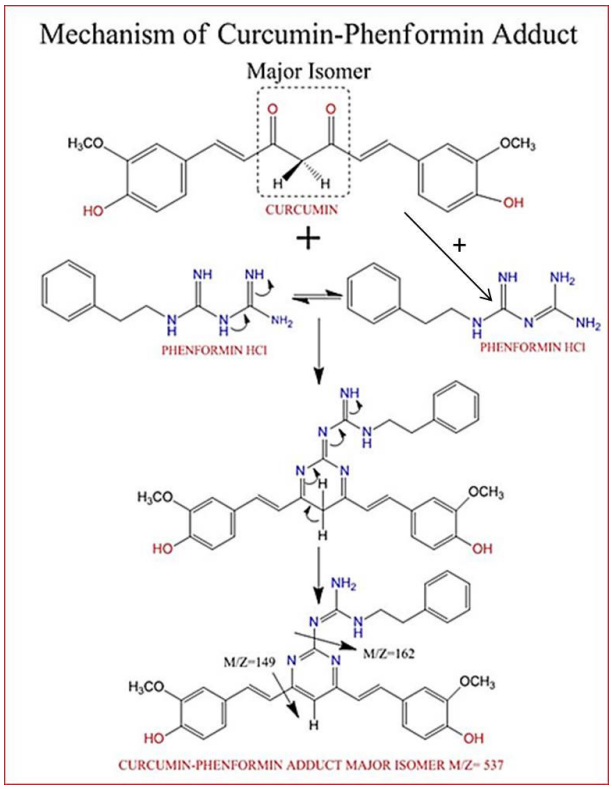

Fig. 8 Reactionmechanism: Curcumin-phenformin adduct (Rf = 0.53, Major Isomer).

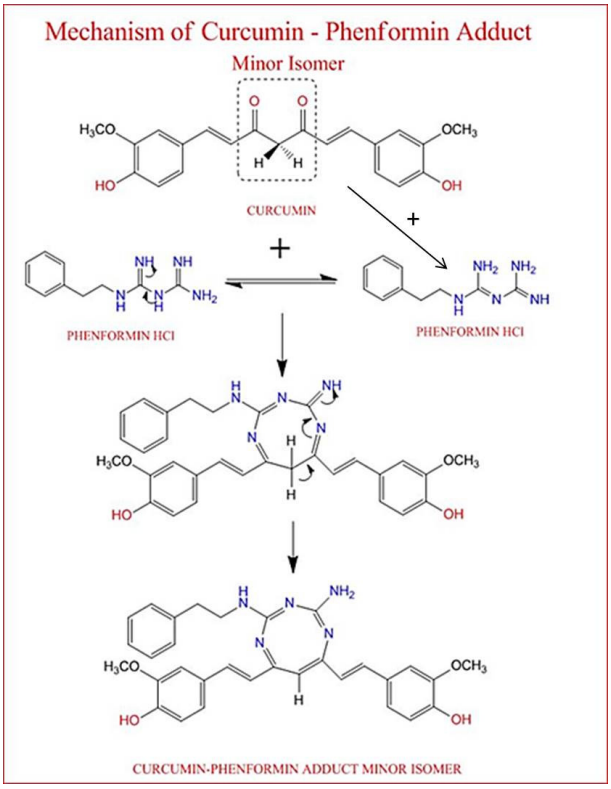

Fig.9 Reactionmechanism: Curcumin-phenformin adduct (Rf = 0.69, minor isomer).

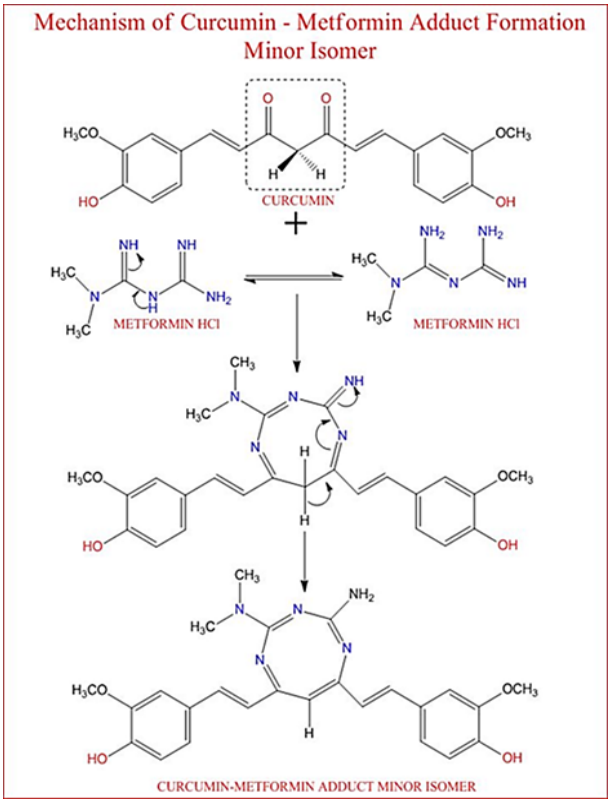

Fig.10 Reactionmechanism: Curcumin-metformin adduct (R_f = 0.69, minor isomer).

that the major isomer according to the reaction mechanism shown in Fig. 8 is thermodynamically more stable than the minor isomer as illustrated by the reaction mechanism in Fig. 9. A plausible proposed reaction mechanism for the formation of eight membered ring of curcumin-metformin adduct described in authors' previous studies is shown in Fig. 10.

This information will be extremely helpful in elucidating the mechanism of inhibition for advanced glycation end products in the individuals suffering from type II diabetes mellitus and various cancers [3, 15-28].

4. Conclusion

Chemical synthesis and identification of major and minor isomers presented in this study may offer novel therapeutic strategies for inhibiting not only AGEs but anti-cancer drugs treatments.

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References

[1] Pernicova, I., Korbonits, M. 2014. "Metformin Mode of Action and Clinical Implications for Diabetes and Cancer." *Nature Reviews Endocrinology* 10: 143-156. doi:10.1038/nrendo.2013.26.

[2] Evans, J. M., Donnelly, L. A., Emslie-Smith, A. M., Alessi, D. R., Morris, A. D. 2005. "Metformin and Reduced Risk of Cancer in Diabetic Patients." *BMJ*. 330:1304-1305.

[3] Miskimins, W. K., Ahn, H. J., Kim, J. Y., Ryu, S., Jung, Y. S., Choi, J. Y. 2014. "Synergistic Anti-Cancer Effect of Phenformin and Oxamate." *PloS ONE*. 9 (1):85576.

[4] Lea, M. A., Chacko, J., Bolikal, S., Hong, J. Y., Chung, R., et al. 2011. "Addition of 2-Deoxyglucose Enhances Growth inhibition but Reverses Acidification in Colon Cancer Cells Treated with Phenformin." *Anticancer* 31: 421-426.

[5] Janzera, A., Germanb, N. J., Gonzalez-Herrerab, K. N., Asarac, J. M., Haigisb, M. C., Struhla, K. 2014. "Metformin and Phenformin Deplete Tricarboxylic Acid Cycle and Glycolytic Intermediates during Cell Transformation and NTPs. in Cancer Stem Cells." *PNAS.* 111 (29): 10574-10579.

[6] Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P., Cerami, A. 1986. "Aminoguanidine Prevents Diabetes-induced Arterial Wall Protein Cross-linking." *Science* 232 (4758): 1629-1632.

[7] Hammes, H. P., Martin, S., Federlin, K., Geisen, K., Brownlee, M. 1991. "Aminoguanidine Treatment Inhibits the Development of Experimental Diabetic Retinopathy." *PNAS.* 88 (24): 11555-11558.

[8] Booth, A. A., Khalifah, R. G., Todd, P., Hudson, B. G. 1997. "In Vitro Kinetic Studies of Formation of Antigenic Advanced Glycation End Products (AGEs). Novel Inhibition of Post-Amadori Glycation Pathways." *J. Biol. Chem.* 272 (9): 5430-5437.

[9] Stitt, A., Gardiner, T. A., Anderson, N. L., Canning, P., Frizzell, N., Duffy, N., et al. 2002. "The AGEs. Inhibitor Pyridoxamine Inhibits Development of Retinopathy in Experimental Diabetes." *Diabetes* 51:2826-2832. doi:10.2337/51.9.2826.

[10] Dayal, B., Mehta, A., Yarramaneni, A. T., Patel, B., Patel, S., O'Connor, P., et al. 2016. "Facile Synthetic Design and Characterization of Curcumin-Metformin Adduct: Potential insights into the Role of This Conjugate in Diseases of Aging." *Environmental Science and Engineering B (JESE. B)* 5: 123-130.

[11] Dayal, B., Gohil, R., Garsondiya, B., Lea, M., Nirujogi, S. P., 2014. "Inhibition of the Formation of Advanced Glycation End Products from Methylglyoxal-modified Ribonuclease by Okra Seed Bioactives" (AGFD. 52) 247th American Chemical Society National Meeting, USA.

[12] Dayal, B., George, R., Lea, M. A. 2013. "Okra Seed Phytochemicals: Potential New Medicines for Glycemic Control." (AGFD. 252) 245th American Chemical Society National Meeting, USA.

[13] Dayal. B., Nirujogi, S. P., Garsondiya, B., Lea, M., Gohil, R. K. 2014. "Efficacy of Okra Seed Extract in Combination with Metformin for Inhibition of Advanced Glycation End Products (AGEs.) in Diabetes-type 2: Potential Implications in Elucidating the Role of Receptor for AGEs. a Key Cellular Target." (AGFD 49) 247th American Chemical Society National Meeting, USA.

[14] Dayal, B., Yannamreddy, V. R., Singh, A. P., Lea, M., Ertel, N. H., "Bioactive Compounds from Okra Seeds: Potential Inhibitors of Advanced Glycation End Products Emerging Trends in Dietary Components for Preventing and Combating Disease." *ACS. Symposium Series* 1093 (16): 287-302. doi: 10.1021/bk-2012-1093.ch016.

[15] Hiroshi, N., 2012. "Cancer Risk in Diabetic Patients Treated with Metformin: A Systematic Review and Meta-analysis." *PloS One* 7(3): 33411.

[16] Zhang, T., Guo, P., Zhan, Y., Xiong, Y., Shan, X., Wang, X., et al. 2013. "The Antidiabetic Drug Metformin Inhibits the Proliferation of Bladder Cancer Cells in Vitro and in Vivo." *Intl. J. Mol. Sci.* 14: 24603-24618.

[17] Rieken, M., Xylinas, E., Kluth, L., Crivelli, J.J., Chrystal, J., Faison, T., et al. 2013."Association of Diabetes Mellitus and Metformin Use with Oncological Outcomes of Patients with non-Muscle-Invasive Bladder Cancer" *BJU Int.* 112 (8): 1105-1112. doi: 10.1111/bju.12448.

[18] Bojkova, B., Orendas, P., Garajova, M, Kassayova, M., Kutna, V., Ahlersova, E., et al. 2009. "Metformin in Chemically-induced Mammary Carcinogenesis in Rats." *Neoplasma* 56: 269-274.

[19] Shackelford, D. B., Abt, E., Gerken, L., Vasquez, D. S., Seki, A., Leblanc, M., et al. 2013. "*LKB1* Inactivation Dictates Therapeutic Response of non-small Cell Lung Cancer to the Metabolism Drug Phenformin." *Cancer Cell* 23 (2): 143-158.

[20] Yuana, P., Ito, K., Perez-Lorenzob, R., Del Guzzo, C., et al. 2013. "Phenformin Enhances the Therapeutic Benefit of BRAFV600E Inhibition in Melanoma." *PNAS.* 110 (45): 18226-18231.

[21] Appleyard, M. V. C., Murray, K. E., Coates, P. J., Wullschleger, S., Bray, S. E., and Kernohan, N. M. 2012. "Phenformin as Prophylaxis and Therapy in Breast Cancer Xenografts." *British Journal of Cancer* 106 (6): 1117-1122.

[22] Walker, R. S., and Linton, A. L. 1959. "Phenethyldiguanide: Dangerous Side-effect." *Brit. M. J.* 2: 1005.

[23] Walker, R. S., Linton, A. L., and Thomson, W. S. T. 1960. "Mode of Action and Side-effects of Phenformin Hydrochloride." *Brit. M. J.* 2: 1567-1569.

[24] Fajans, S. S., Moorhouse, J. A., Doorenbos, H., Louis, L. H., and Conn, J. W. 1960. "Metabolic Effects of Phenethylbiguanide in Normal Subjects and in Diabetic Patients." *Diabetes* 9: 194-201.

[25] Lea, M. A., Guzman, Y., and DesBordes, C. 2016. "Inhibition of Growth by Combined Treatment with Inhibitors of Lactate Dehydrogenase and either Phenformin or Inhibitors of 6-Phosphofructo- 2-kinase/ fructose-2,6-bisphosphatase-3." *Anticancer Res*. 36 (4):1479-88.

[26] Zhuang, Y., and Miskimins, W. K. 2011. "Metformin Induces both Caspase-dependent and Poly (ADP-ribose) Polymerase-dependent Cell Death in Breast Cancer Cells." *Mol. Cancer Res.* 9:603-15.

[27] Cheong, J. H., Park, E. S., Liang, J., Dennison, J. B.,

Tsavachidou D. et al., 2011. "Dual Inhibition of Tumor Energy Pathway by 2-Deoxyglucose and Metformin is Effective Against a Broad Spectrum of Preclinical Cancer Models." *Mol Cancer Ther.* 10: 2350-2362.

[28] Janzera, A., Germanb, N. J., Gonzalez-Herrerab, K. N., Asarac, J. M., Haigisb, M. C. and Struhla, K. 2014. "Metformin and Phenformin Deplete Tricarboxylic Acid Cycle and Glycolytic Intermediates during Cell Transformation and NTPs in Cancer Stem Cells". *PNAS* 111 (29): 10574-10579.

[29] Anand, P., Thomas, S. G., Kunnumakkara, A. B., Sundaram, C., Harikumar, K. B., Sung, B., et al. 2008. "Biological Activities of Curcumin and its Analogues (Congeners) Made by Man and Mother Nature." *Biochem. Pharmacol.* 76: 1590-1611.

[30] Kim, H. J., Young, P. J. 2009. "Direct Analysis of Curcumin in Turmeric by DART-MS." *Phytochem. Anal.* 20: 372-377.

[31] Dayal, B., Swayam, N. P., Gohil, R., Garsondiya, B., and Lea, M. 2014. "Separation of Curcuminoids in Turmeric by Argentation Thin-layer Chromatography and their Comparative Antidiabetic Activity with Okra Seed Extract." 248th American Chemical Society National Meeting, San Francisco, CA August.

[32] Park, W., Amin, A. R., Chen, Z. G., and Shin, D. M. 2013. "New Perspectives of Curcumin in Cancer Prevention." *Cancer Prev. Res.* 6: 387-400.

[33] Howells, L. M., Mahale, J., Sale, S., McVeigh, L., Steward, W., Thomas, A., and Brown, K. 2014. "Translating Curcumin to the Clinic for Lung Cancer Prevention: Evaluation of the Preclinical Evidence for its Utility in Primary, Secondary, and Tertiary Prevention Strategies." *J. Pharmacol Exp. Ther.* 350: 483-494.

[34] Gohil, R., Lea, M. A., DesBordes, C., and Dayal, B. 2014. "Inhibitory Effects of Turmeric Extracts and Curcumin on Proliferation and Differentiation of Human Colon Cancer Cells." 248th American Chemical Society National Meeting, San Francisco, CA.

[35] Dayal, B., Mehta, A., Kulkarni, D., Pandey, A., Li, S. G., and Lea, M. A. 2015. "Green Synthesis and Electrospray Atmospheric Pressure Ionization Mass Spectral Studies of Hydrazinocurcumin: A Potentially Important Derivative for Intestinal Absorption Studies at the Nanolevel." (AGFD 69) 249th American Chemical Society National Meeting, Denver, Colorado.

[36] Chin, D., Huebbe, P., Pallauf, K., and Rimbach, G. 2013. "Neuroprotective Properties of Curcumin in Alzheimer's Disease—merits and Limitations." *Curr. Med. Chem.* 20: 3955-3985.

[37] Narlawar, R., Pickhardt, M., Leuchtenberger, S., Baumann, K., Krause, S., Dyrks, T., and Schmidt, B. 2008. "Curcumin-derived Pyrazoles and Isoxazoles: Swiss Army Knives or Blunt Tools for Alzheimer's Disease?" *Chem. Med. Chem.* 3 (1): 165-172.

[38] Hafner-Bratkovič, I., Gašperšič, J., Šmid, L. M., Bresjanac, M., and Jerala, R. 2008. "Curcumin Binds to the α-helical Intermediate and to the Amyloid form of Prion Protein—a New Mechanism for the Inhibition of PrPSc. accumulation." *J. Neurochem.* 104 (6): 1553-1564.

[39] Brondino, N., Re, S., Boldrini, A., Cuccomarino, A., Lanati, N., Barale, F., et al. 2014. "Curcumin as a Therapeutic Agent in Dementia: a Mini Systematic Review of Human Studies." *Sci. World J.* 22: 2014:174282.

[40] Lopresti, A. L., Maes, M., Maker, G. L., Hood, S. D. and Drummond, P. D. 2014. "Curcumin for the Treatment of Major Depression: a Randomised, Double-blind, Placebo Controlled Study." J. Affect. Disord. 167: 368-375.