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A systematic literature review of fenugreek seed toxicity by using ToxRTool: evidence from preclinical and clinical studies

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Abstract

Fenugreek (*Trigonella foenum graecum*) seed extract is a bioactive ingredient of many food supplements. Hence, there is a need for systematic assessment of the quality of published toxicological studies for its use in human health, hazard consideration, and risk assessment. The aim of the present investigation was to determine the reliability of published toxicological studies of fenugreek seed by using ToxRTool (Toxicological data reliability assessment tool). A comprehensive systematic literature search was conducted in PubMed, EMBASE, Cochrane Library, CPCI-S, ICTRP, Ovid, and Google Scholar till October 2018. Each identified study was evaluated for its quality using the ToxRTool with outcomes such as combined score, weighted score, and reliability category by three independent raters. Correlations of various criteria groups with the combined score were evaluated by Pearson correlation and Kendall rank correlation coefficient. Inter-rater consistency was measured by Cronbach's alpha coefficient. The database searches initially yielded 436 results, of which 391

(89.67%) studies were “not assignable”. The remaining 45 studies were included for quantitative analysis by ToxRTool. Based on the weighted score, 17 *in-vivo*, and 3 *in-vitro* studies were determined to be “Reliable Without Restriction” which were conducted according to international guidelines such as GLP. These studies have a significant difference ($p < 0.05$) for the combined and weighted score as compared to non-GLP studies. Remaining 28 *in-vivo* and 2 *in-vitro* studies were determined to be “Not Reliable.” The GLP studies conducted with “identified study material” have a significant difference ($p < 0.0001$) between combined and weighted score as compared to studies which used “non-identified study material”. For criteria group of ToxRTool I, III and V, the Pearson correlation with the combined score was found to be 0.875, 0.734 and 0.905, respectively and Kendall rank correlation coefficient was found to be 0.764, 0.551 and 0.752, respectively. Cronbach’s alpha coefficient for combined score and weighted score were 0.920 and 0.887, respectively. In conclusion, the ToxRTool was found useful to identify seventeen toxicity studies of fenugreek seeds as “Reliable without Restrictions”. These studies showed a broad margin of safety for the standardized extract of fenugreek seeds and can form a basis for toxicological risk assessment with reasonable certainty.

Keywords: Food science, Nutrition, Food safety

1. Introduction

In the recent times, the problem of food safety is gaining importance in developed and developing countries. Major causes that are responsible for food toxicity include poor knowledge, malpractice during their preparation, the presence of bacteria, toxins or allergens in the food, and cross-contamination with harmful organisms [1]. Many short-term (nausea, vomiting, weakness, diarrhea, mild fever and headache) and long-term (kidney failure, brain and nerve damage) health hazards may result from exposure to food toxicants. Therefore, efforts are being taken by various governmental and regulatory agencies (such as World Health Organization International Program on Chemical Safety, US Food and Drug Administration, US Environmental Protection Agency, Agency for Toxic Substances and Disease Registry, European Chemicals Agency, etc.) to publish evidences based on peer-reviewed scientific literature related to health assessments of chemicals.

Fenugreek (*Trigonella foenum graecum* L., family: Fabaceae) is one of the most promising traditional medicinal plants cultivated widely in India, Egypt, and Middle Eastern countries [2]. Its leaves and seeds have been extensively used as medicine, spice, and vegetable in various pharmaceutical, nutraceutical, and functional food industries [3]. Researchers have investigated multifaceted therapeutic benefits of fenugreek seeds against a variety of ailments including diabetes, cancer,

hyperlipidemia, inflammation, neurotoxicity, hepatotoxicity, ulcers, wound, bacterial and fungal infections, weakness, and edema of the legs [4, 5]. Apart from its beneficial effects, many studies have also documented its toxicological profile [6, 7, 8]. However, these published literature are diverse in nature. Although most studies suggested that its utilization is safe, some reported toxicity to the gastrointestinal and reproductive system. Additionally, researchers have published studies summarizing “evidence-based toxicology” results of fenugreek [9, 10]. However, the quality and reliability of toxicological studies of fenugreek were not assessed in the past. Furthermore, some of the reviews based on toxicological studies are diverse with respect to part of the fenugreek plant (whole, leaves, seeds), route (oral, intra-peritoneal) administration, etc. These variations had a major impact on scientific conclusions and interpretations of these reviews. It may create confusion among the public regarding the safety of fenugreek seeds-based healthcare products.

To make a stronger and more reliable risk assessment on scientific basis, critical appraisal of reviews of scientific studies is needed. For such risk assessment, each evaluation of toxicological study needs to be based on standardized criteria, specifically designed for such purposes [11]. In this view, National Academy of Sciences perceived the need to use standard criteria for the assessment of study quality of toxicological studies providing transparency and consistency of risk assessments [12]. Additionally, experts from REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) and Society of Toxicology (SOT) suggested rating of the toxicological studies for quality and reliability during regulatory decisions [13, 14]. Thereafter, researchers have developed a reliable and objective assessment tool “ToxRTool” (**T**oxicological data **R**eliability Assessment **T**ool) to evaluate the quality of published toxicological studies [15]. It is a validated, standardized, reproducible, and widely used tool that code toxicological studies based on reliability criteria [15]. The identified studies using ToxRTool are “reliable” and can be included in a human health hazard assessment.

To the best of our knowledge, no systematic study has been carried out to determine the toxicological profile of fenugreek seed using an assessment tool like ToxRTool. Therefore, the present analysis was aimed to evaluate systematic evaluation of existing toxicological studies of fenugreek seed for their reliability and quality by using the ToxRTool.

2. Materials and methods

This systematic review was conducted in line with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement [16].

2.1. Search strategy

We systematically searched the published scientific literature concerning toxicological studies on fenugreek seeds that are in English language. The literature identification process was performed by searching the following electronic databases: PubMed, EMBASE, the Cochrane Library, CPCI-S (Conference Proceedings Citation Index-Science), ICTRP (International Clinical Trials Registry Platform), Ovid, and Google Scholar for relevant publications by using Medical Subject Headings (MeSH) terms. These were systematically searched for titles and abstracts published between inception date of the database and October 2018 (Table 1). Spelling variations were also used. Additional articles were obtained through citation snowballing to locate primary sources. We also searched Clinicaltrials.gov to identify such studies. The results of this search identified additional relevant publications detailing primary research on the toxicity of fenugreek seed to be included in this review. Finally, references within the publications identified in the first two steps were reviewed to determine if any other relevant publications were overlooked, and a few unidentified publications were added to the list of publications to be evaluated as part of this review.

2.2. Study inclusion criteria

Titles and abstracts were identified as potentially eligible by two independent reviewers [PT, AK]. Titles and abstracts meeting the following inclusion criteria were selected for full-article (primary literature) review:

- English language article
- Studies reporting the toxicological profile of fenugreek seed (in the form of its extract, in the feed or isolated compound from fenugreek seed)
- Outcome: toxicity endpoints

2.3. Exclusion criteria

The exclusion criteria were as follows: (1) studies where species were treated with fenugreek leaf/root/plant/flower (2) studies where species were treated with fenugreek seeds combined with other test compound (3) studies reporting only the chemistry of fenugreek seeds and no biological testing (e.g. phytochemistry work, analytical work) (4) studies reporting the protective action and/or mechanism of fenugreek seed against drug or disease induced toxicity (5) studies with no toxicity endpoints reported (6) secondary literature such as letters, reviews, editorials, commentary conference presentations, proceedings, abstracts, magazine, handbooks or white papers, (7) case series (sample size <10 patients) or case reports (8) pharmacodynamic/pharmacokinetic studies, and (9) studies with full-text published in a language other than English. Review articles were excluded as well - however - their

Table 1. The detailed Search strategy.

Sr. No.	Searches	Results
The detailed Search strategy for PubMed (((“trigonella”[MeSH Terms] OR “trigonella”[All Fields] OR “fenugreek”[All Fields]) AND (“seeds”[MeSH Terms] OR “seeds”[All Fields] OR “seed”[All Fields])) OR (((“trigonella”[MeSH Terms] OR “trigonella”[All Fields] OR “trigonella”[All Fields] AND “foenum”[All Fields] AND “graecum”[All Fields]) OR “trigonella foenum graecum”[All Fields]) AND (“seeds”[MeSH Terms] OR “seeds”[All Fields] OR “seed”[All Fields])) OR (((“trigonella”[MeSH Terms] OR “trigonella”[All Fields] OR “trigonella”[All Fields] AND “foenum”[All Fields] AND “graecum”[All Fields]) OR “trigonella foenum graecum”[All Fields]) AND (“seeds”[MeSH Terms] OR “seeds”[All Fields] OR “seed”[All Fields])) OR (“trigonella”[MeSH Terms] OR “trigonella”[All Fields])) AND (((“adverse effects”[Subheading] OR (“adverse”[All Fields] AND “effects”[All Fields]) OR “adverse effects”[All Fields]) OR (“toxicity”[Subheading] OR “toxicity”[All Fields]) OR (“poisoning”[Subheading] OR “poisoning”[All Fields] OR “poisoning”[MeSH Terms]) OR (Adverse[All Fields] AND event[All Fields])) AND (((“humans”[MeSH Terms] OR “humans”[All Fields]) OR (“humans”[MeSH Terms] OR “humans”[All Fields] OR “human”[All Fields]) OR (“rats”[MeSH Terms] OR “rats”[All Fields] OR “rat”[All Fields]) OR (“mice”[MeSH Terms] OR “mice”[All Fields]) OR (“mice”[MeSH Terms] OR “mice”[All Fields] OR “mouse”[All Fields]))		
The detailed Search strategy for Ovid		
1.	Fenugreek seed.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	339
2.	<i>Trigonella foenum-graecum</i> seed.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	72
3.	<i>Trigonella foenum graecum</i> seed.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	72
4.	Trigonella.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	1225
5.	Adverse effects.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	1972932
6.	Toxicity.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	1014856
7.	Poisoning.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	175823
8.	Adverse event.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	107192
9.	(Humans or Human).mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	20020778
10.	(mice or mouse).mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	2459639
11.	rat.mp. [mp=ti, ab, tx, ct, sh, ot, nm, hw, fx, kf, px, rx, an, ui, sy]	1353776
12.	1 or 2 or 3 or 4	1287
13.	5 or 6 or 7 or 8	3012637
14.	9 or 10 or 11	21982722
15.	12 and 13 and 14	216
16.	Remove duplicates from 15	203
17.	Limit 16 to English language	201

reference lists were searched for eligible articles. All retrieved full-text articles were again independently reviewed by the three reviewers (PAT, ADK, PW), according to the same predefined inclusion and exclusion criteria. When consensus regarding the eligibility could not be accomplished, an agreement was obtained through discussions with a fourth arbiter (SLB).

2.4. Quality assessment of the articles

The quality of each study included in the analysis was assessed using the “ToxR-Tool” [15]. Three independent reviewers (PAT, ADK, PW) with risk assessment experience rated each publication of fenugreek seed involving toxicity testing. One of the raters (PW) is a qualified toxicologist and pathologist. Other two reviewers had participated in many GLP complaint and published toxicological studies on dietary ingredients in peer-reviewed journals. All the three reviewers were familiar with the ToxRTool before participation in this project. Each rater evaluated the articles using the ToxRTool downloaded from the European Commission’s Joint Research Center website [17]. The raters used the *in-vitro* ToxRTool spreadsheet to indicate whether 18 criteria were met in the following 5 areas: (1) test substance identification, (2) test system characterization, (3) study design description, (4) study results documentation, and (5) plausibility of study design and data. For *in-vivo* studies, the raters used a separate spreadsheet that listed 21 criteria in the same 5 areas that were used for *in-vitro* studies, except ‘test system characterization’ which is used for *in-vitro* studies instead of ‘test organism characterization’ that is used for *in-vivo* studies. The raters assigned score for each criterion on the appropriate spreadsheet as either a ‘1’ (‘yes-met’) or a ‘0’ (‘no-not met’).

2.5. Combined score, mean combined score, and standard deviation

A combined score was calculated for each rater and each study by summing the scores for all criteria for each study. A mean combined score (the average of all raters’ combined scores), and the standard deviation were also calculated.

2.6. Weighted score, mean weighted score, and standard deviation

A weighted score was calculated for each rater and study by summing the scores for all criteria (in which sum of scores only for red colored criteria was considered). The mean weighted scores (the average of weighted score of all raters), and standard deviation were also calculated.

2.7. Reliability categorization

Reliability categorization of each study, based on the mean combined score and mean weighted score, was carried out according to a previously reported method [18]. Based on the combined score, the ToxRTool assigned each study a reliability category 1 (reliable without restrictions), 2 (reliable with restrictions), 3 (not reliable). In determining the Weighted Score categorization, the ToxRTool identified certain criteria as critical. For this classification, if any individual ‘critical’ criterion was not met, a study could not be assigned as reliable, regardless of the study’s Numerical Score (Table 2).

Table 2. Numerical score and weighted score reliability categorization.

Categorization	Total score (<i>in-vivo</i>)	Total score (<i>in-vitro</i>)	Consideration	(Proposed) consequence
1	18–21	15–18	Reliable without restrictions	Useful, check relevance for intended purpose
2	13–17	11–14	Reliable with restrictions	Potentially useful, check relevance for the intended purpose
3	<13 or not all red criteria met	<11 or not all red criteria met	Not reliable	Generally, not to be used as key study, but depending on the shortcomings of the study it may still be useful in weight-of-evidence approaches or as supportive information
4			Not assignable: documentation insufficient (reviews, handbooks, other secondary sources)	Generally, not to be used as a key study, but depending on the shortcomings of the study it may still be useful in weight-of-evidence approaches or as supportive information. (This category is not an outcome of this evaluation tool)

2.8. Calculating inter-rater consistency for each criterion

To determine which criteria had low agreement across raters and were therefore problematic, an inter-rater consistency value was calculated for each criterion. The Cronbach’s Alpha coefficient was selected to measure inter-rater consistency, as each performs reasonably well in the presence of high and low agreement.

2.9. Data analysis

Data were analyzed by using the SPSS software. The data of numerical score and weighted score were presented as the mean and standard deviation (SD). The internal

consistency and reliability of the factors were evaluated using the Cronbach's Alpha. The data of administered dosage form and guideline followed were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple range tests for post hoc analysis. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Summary of included studies

As shown in Fig. 1 (PRISMA flow chart), the database searches for “fenugreek seed toxicity” initially yielded 436 results. After duplicates were removed and reports screened by title, keywords, and abstract, they were screened for inclusion and exclusion. After screening of title or abstract, 376 of these (Case Study/Series (2), Chemistry of fenugreek seed (77), Conference presentation (11), Copy/duplicate (1), In combination with other drugs (22), No toxicity endpoints reported (23), Parts other than seeds (8), Protective action against toxicity (200) and Review/editorial (32)) were excluded. Further, based on the full-text screening, 15 studies (In combination with other drugs (4) and no toxicity endpoints reported (11)) were excluded. According to the Klimisch categories of ToxRTool, the category code of these 391 (89.67%) studies were “4” which indicates that a paper is “not assignable” because it does not contain any primary data. Remaining 45 (10.33%) studies were included for quantitative analysis by ToxRTool [6, 7, 8, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59].

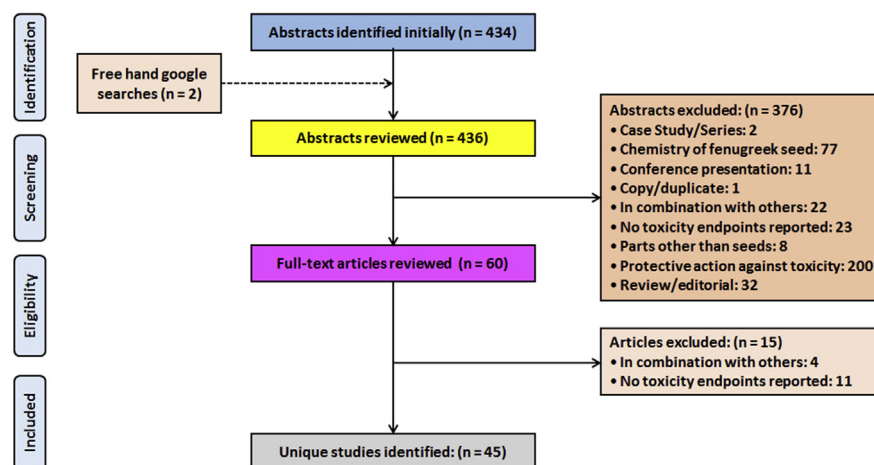


Fig. 1. PRISMA flow diagram depicting the selection of studies for systematic review for ToxRTool evaluation.

The primary findings from the included *in-vivo* and *in-vitro* studies are summarized in Tables 3 and 4. The assignable toxicology publications on fenugreek seeds

Table 3. Characteristics of included *In-vivo* studies.

Sr. No.	Authors	Study conducted according to recent guidelines	Species/test system used	Dose of test compound	Duration of exposure of test compound (in days)	Isolated/extract/ powder/diet
1	Ahirwar et al. (2010)	No	Wistar rats	500 mg/kg	7 days	Extract
2	Al-Ashban et al. (2010)	Yes (W.H.O. protocol, 1967)	Swiss albino mice	Acute dosages: 0.5, 1.0 and 3 g/kg Chronic dosage: 100 mg/kg	Acute (24 h) and chronic (90 days)	Extract
3	Al-Shaikh et al. (1999)	No	Goat	25 or 50% fenugreek	63 days	Diet
4	Al-Yahya (2013)	No	Swiss albino Mice	153, 305 and 610 mg/kg	90 days	Capsules
5	Aswar et al. (2010)	No	Wistar rats	10 and 35 mg/kg	28 days	Isolated
6	Aswar et al. (2008)	Yes (OECD-425)	Wistar rats and Swiss albino Mice	10 and 35 mg/kg	28 days	Isolated
7	Aswar et al. (2009)	No	Wistar rats	75 mg/kg	7 days	Isolated
8	Awad et al. (2015)	No	Fish	10 (1%), 50 (5%) or 100 (10%) g/kg	28 days	Diet
9	Bin-Hafeez et al. (2003)	No	Swiss albino mice	50, 100 and 250 mg/kg	10 days	Extract
10	Chevassus et al. (2009)	Yes (ICH-GCP)	Human subject	588 mg (app. 8 mg/kg)	14 days	Dry hydro-alcoholic extract in tablets
11	Choudhary et al. (2001)	No	Swiss albino mice	1, 2, 5 and 10%	28 days	Diet
12	Dande and Patil (2012)	Yes (OECD-423)	Wistar rats	2000 mg/kg	1 day	Saponin extract
13	Deshpande et al. (2017a)	Yes (OECD-423 and OECD-408)	Wistar rats	AOT: 300 and 2000 mg/kg 90-day repeated dose toxicity: 250, 500, and 1000 mg/kg	For AOT: Single day For 90-day repeated dose toxicity: 90 days	Isolated
14	Deshpande et al. (2016a)	Yes (OECD-414)	Wistar rats	250, 500 and 1000 mg/kg	20 days	Isolated
15	Deshpande et al. (2016b)	Yes (OECD-414)	Wistar rats	250, 500 and 1000 mg/kg	20 days	Isolated
16	Deshpande et al. (2017b)	Yes (OECD-414)	Wistar rats	250, 500 and 1000 mg/kg	20 days	Isolated

(continued on next page)

Table 3. (Continued)

Sr. No.	Authors	Study conducted according to recent guidelines	Species/test system used	Dose of test compound	Duration of exposure of test compound (in days)	Isolated/extract/ powder/diet
17	Deshpande et al. (2016c)	Yes (OECD-423 and OECD-408)	Wistar rats	AOT: 2000 mg/kg For 90-day repeated dose toxicity: 250, 500, and 1000 mg/kg	For AOT: Single day For 90-day repeated dose toxicity: 90 days	Isolated
18	Deshpande et al. (2017c)	Yes (OECD-423 and OECD-408)	Wistar rats	AOT: 2000 mg/kg For 90-day repeated dose toxicity: 250, 500, and 1000 mg/kg	For AOT: Single day For 90-day repeated dose toxicity: 90 days	Isolated
19	Deshpande et al. (2016d)	Yes (OECD-423 and OECD-408)	Sprague-Dawley rats	AOT: 2000 mg/kg For 90-day repeated dose toxicity: 250, 500, and 1000 mg/kg	For AOT: Single day For 90-day repeated dose toxicity: 90 days	Isolated
20	Effraim et al. (1999)	No	Wistar rats	1, 1.5 and 2 g/kg	28 days	Extract
21	Flammang et al. (2004)	No	BR mice	500, 1000 or 2000 mg/kg/day	3 days	Extract
22	Folwarczna et al. (2014a)	No	Wistar rats	50 mg of 4-hydroxy-L-isoleucine/kg	28 days	Diet
23	Folwarczna et al. (2014b)	No	Wistar rats	50 mg/kg	28 days	Isolated
24	Kandhare et al. (2015)	Yes (OECD-425 and OECD-407)	Swiss albino mice	AOT: 55, 175, 550, 1750 and 5000 mg/kg, Repeated dose 28-day oral toxicity: 250, 500, 1000 mg/kg	For AOT: Single day For 28-day repeated dose toxicity: 28 days	Isolated
25	Kandhare et al. (2016a)	Yes (OECD-425 and OECD-407)	Swiss albino mice	AOT: 55, 175, 550, 1750 and 5000 mg/kg, Repeated dose 28-day oral toxicity: 37.5, 75, 150 mg/kg	For AOT: Single day For 28-day repeated dose toxicity: 28 days	Isolated
26	Kassem et al. (2006)	No	New Zealand white rabbits	Fenugreek seed powder diet (30%)	90 days	Diet
27	Khalki et al. (2013)	No	Swiss CD1 mice	1 g/kg/day	Gestation period of mice is usually 19–21 days	Extract
28	Khalki et al. (2010)	No	Swiss albino mice	500 and 1000 mg/kg	Gestation period of mice is usually 19–21 days	Extract
29	Maheshwari et al. (2017)	Yes (ICH-GCP)	Human subject	500 mg/day	90 days	Isolated
30	Majumdar et al.	No	Wistar rats	0.25 g/kg	28 days	Isolated

(continued on next page)

Table 3. (Continued)

Sr. No.	Authors	Study conducted according to recent guidelines	Species/test system used	Dose of test compound	Duration of exposure of test compound (in days)	Isolated/extract/ powder/diet
	(2017)					
31	Mokashi et al. (2014)	No	Human subject	600 mg	Acute Study	Isolated
32	Panda et al. (1999)	No	Swiss albino mice	0.11 g/kg	15 days	Extract
33	Petit et al. (1995)	No	Wistar rats	12.5 mg/300 g	28 days	Isolated
34	Poole et al. (2010)	No	Human subject	500 mg	56 days	Isolated
35	Rao et al. (2015)	No	Human subject	600 mg	Over two menstrual cycles	Isolated
36	Rao et al. (2016)	No	Human subject	600 mg	90 days	Extract
37	Sharma (1986)	No	Human subject	25 g	Acute	Extract
38	Steels et al. (2011)	No	Human subject	600 mg	42 days	Isolated
39	Swaroop et al. (2014)	Yes (OECD-407 and OECD-471)	Wistar rats and Swiss albino Mice	Acute: 0, 500, 1000 and 2000 mg/kg 28-day: 0, 250, 500 and 1000 mg/kg	AOT: Single day 28-day repeated dose toxicity: 28 days	Isolated
40	Wankhede et al. (2016)	Yes (Guidelines of Helsinki Declaration)	Human subject	600 mg	56 days	Isolated
41	Wilborn et al. (2010)	No	Human subject	500 mg	56 days	Isolated
42	Mowla et al. (2009)	No	Wistar rats	3 g/kg	Acute study	Extract
43	Hamad et al. (2017)	No	Swiss albino mice	3, 6, and 9 g/kg	Acute study	Extract
44	Wilborn et al. (2017)	No	Human subject	450 mg	14 days	Isolated
45	Hamad et al. (2017)	Yes	Swiss albino mice	3, 6, and 9 g/kg	Acute study	Extract

AOT: Acute Oral Toxicity; ICH-GCP: International Conference on Harmonization-Good Clinical Practice; OECD: Organization for Economic Co-operation and Development.

Table 4. Characteristics of included *In-vitro* studies.

Sr. No.	Authors	Study conducted according to recent guideline	Species/Test system used	Dose of test compound
1	Deshpande et al. (2017a)	Yes (OECD-471 and OECD-473)	<i>Salmonella typhimurium</i>	50, 150, 500, 1,500, and 5,000 µg/plate
2	Deshpande et al. (2016c)	Yes (OECD-471)	<i>Salmonella typhimurium</i>	5000.00, 1666.67, 555.55, 185.18 and 61.72 µg/plate
3	Deshpande et al. (2017c)	Yes (OECD-471)	<i>Salmonella typhimurium</i>	5000.00, 1666.67, 555.55, 185.18 and 61.72 µg/plate
4	Flammang et al. (2004)	No	<i>Salmonella typhimurium</i>	33.3, 100, 333, 1000, 3330, 5000 µg/plate
5	Swaroop et al. (2014)	Yes (OECD-471)	<i>Salmonella typhimurium</i>	5000, 1500, 500, 150 and 50 µg/plate

OECD: Organization for Economic Co-operation and Development.

utilized a variety of forms of fenugreek seed viz., powder of fenugreek seed, various extracts of fenugreek seeds, fenugreek seeds in diet/feed, and isolated phytoconstituents from fenugreek seed. Twenty-four studies were conducted on the isolated phytoconstituents, 16 studies used fenugreek seed extract, and 5 studies used fenugreek seeds that was included in the diet/feed (Table 3).

3.2. Inter-rater consistency reliability

The inter-rater consistency was measured by Cronbach's alpha coefficient amongst the three raters viz. PAT, ADK, PW for scoring the ToxRTool. For combined score, the inter-rater consistency reliability was 0.920 which means there was 92.0% agreement between the raters. Whereas for weighted score, inter-rater consistency reliability was 0.887 which means there was 88.7% agreement between the raters.

3.3. Reliability categorizations

Tables 5 and 6 summarize the ToxR reliability assessment score for individual *in-vivo* and *in-vitro* toxicity studies, respectively. For the 45 evaluated *in-vivo* studies, 23 were determined to be "Reliable Without Restriction" (51%), 13 determined to be "Reliable With Restriction" (29%), and 9 were determined to be "Not Reliable" (20%) based on their initial category score. However, checking of red scores (weighted scores) lead to a revision in the categories which now contained 17 studies under "Reliable Without Restriction" (38%), and 28 studies under "Not Reliable" (62%) (Fig. 2).

Of the five evaluated *in-vitro* studies, four were determined to be "Reliable Without Restriction" (80%), and one was determined to be "Reliable With Restrictions," (20%). However, when they were checked for their red scores (revised category),

Table 5. ToxR reliability assessment scores for individual *in-vivo* toxicity studies.

Sr. No.	Authors	Criteria group I (Mean $\pm$ SD)	Criteria group II (Mean $\pm$ SD)	Criteria group III (Mean $\pm$ SD)	Criteria group IV (Mean $\pm$ SD)	Criteria group V (Mean $\pm$ SD)	Combined score (Mean $\pm$ SD)	Weighted score (Mean $\pm$ SD)	Initial category (Based on mean combined score)	Revised category (Based on mean weighted score)
1	Ahirwar et al. (2010)	0.00 $\pm$ 0.00	5.00 $\pm$ 0.00	5.00 $\pm$ 1.00	1.67 $\pm$ 0.57	0.00 $\pm$ 0.00	11.67 $\pm$ 1.52	5.00 $\pm$ 1.00	3	3
2	Al-Ashban et al. (2010)	1.33 $\pm$ 0.57	5.00 $\pm$ 0.00	5.33 $\pm$ 0.577	1.67 $\pm$ 0.57	0.67 $\pm$ 1.15	14.00 $\pm$ 2.64	6.67 $\pm$ 1.15	2	3
3	Al-Shaikh et al. (1999)	0.33 $\pm$ 0.57	3.67 $\pm$ 0.57	4.00 $\pm$ 3.46	2.33 $\pm$ 0.57	1.33 $\pm$ 0.57	11.66 $\pm$ 3.21	6.33 $\pm$ 0.57	3	3
4	Al-Yahya (2013)	1.00 $\pm$ 1.00	5.00 $\pm$ 0.00	5.33 $\pm$ 1.52	2.67 $\pm$ 0.57	1.00 $\pm$ 1.00	15.00 $\pm$ 4.00	6.33 $\pm$ 1.52	2	3
5	Aswar et al. (2010)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	5.33 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	19.33 $\pm$ 0.57	7.33 $\pm$ 0.57	1	3
6	Aswar et al. (2008)	1.67 $\pm$ 0.57	5.00 $\pm$ 0.00	4.67 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	16.33 $\pm$ 0.57	6.33 $\pm$ 0.57	2	3
7	Aswar et al. (2009)	2.67 $\pm$ 0.57	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	18.67 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
8	Awad et al. (2015)	1.67 $\pm$ 0.57	4.33 $\pm$ 0.57	6.33 $\pm$ 0.57	2.67 $\pm$ 0.57	1.00 $\pm$ 0.00	16.00 $\pm$ 0.00	7.00 $\pm$ 0.00	2	3
9	Bin-Hafeez et al. (2003)	2.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	18.00 $\pm$ 0.00	7.33 $\pm$ 0.57	1	3
10	Chevassus et al. (2009)	3.67 $\pm$ 0.57	5.00 $\pm$ 0.00	5.67 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	19.33 $\pm$ 0.57	7.67 $\pm$ 0.57	1	3
11	Choudhary et al. (2001)	0.67 $\pm$ 0.57	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	1.00 $\pm$ 0.00	15.67 $\pm$ 0.57	6.33 $\pm$ 0.57	2	3
12	Dande and Patil (2012)	1.67 $\pm$ 1.52	4.67 $\pm$ 0.57	4.33 $\pm$ 1.52	1.33 $\pm$ 1.52	0.33 $\pm$ 0.57	12.33 $\pm$ 5.50	6.00 $\pm$ 2.00	3	3
13	Deshpande et al. (2017a)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.67 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.67 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
14	Deshpande et al. (2016a)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.67 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.67 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1

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Table 5. (Continued)

Sr. No.	Authors	Criteria group I (Mean $\pm$ SD)	Criteria group II (Mean $\pm$ SD)	Criteria group III (Mean $\pm$ SD)	Criteria group IV (Mean $\pm$ SD)	Criteria group V (Mean $\pm$ SD)	Combined score (Mean $\pm$ SD)	Weighted score (Mean $\pm$ SD)	Initial category (Based on mean combined score)	Revised category (Based on mean weighted score)
15	Deshpande et al. (2016b)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.67 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.67 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
16	Deshpande et al. (2017b)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.67 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.67 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
17	Deshpande et al. (2016c)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.67 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.67 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
18	Deshpande et al. (2017c)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.33 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.3.00 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
19	Deshpande et al. (2016d)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.33 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.33 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
20	Effraim et al. (1999)	1.00 $\pm$ 0.00	4.33 $\pm$ 0.57	5.67 $\pm$ 0.57	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	11.00 $\pm$ 1.00	5.67 $\pm$ 0.57	3	3
21	Flammang et al. (2004)	1.33 $\pm$ 1.15	4.33 $\pm$ 0.57	6.00 $\pm$ 0.00	2.67 $\pm$ 0.57	0.67 $\pm$ 1.15	15.00 $\pm$ 3.00	7.00 $\pm$ 1.00	2	3
22	Folwarczna et al. (2014a)	3.67 $\pm$ 0.57	4.67 $\pm$ 0.57	6.00 $\pm$ 0.00	2.33 $\pm$ 1.15	0.67 $\pm$ 0.57	17.33 $\pm$ 2.08	6.67 $\pm$ 0.57	2	3
23	Folwarczna et al. (2014b)	4.00 $\pm$ 0.00	4.67 $\pm$ 0.57	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	19.67 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
24	Kandhare et al. (2015)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.00 $\pm$ 0.00	8.00 $\pm$ 0.00	1	1
25	Kandhare et al. (2016a)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.00 $\pm$ 0.00	8.00 $\pm$ 0.00	1	1
26	Kassem et al. (2006)	1.67 $\pm$ 1.52	3.33 $\pm$ 1.15	4.33 $\pm$ 2.08	3.00 $\pm$ 0.00	0.00 $\pm$ 0.00	12.33 $\pm$ 4.72	5.67 $\pm$ 2.30	3	3
27	Khalki et al. (2013)	3.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	2.67 $\pm$ 0.57	0.67 $\pm$ 1.15	17.33 $\pm$ 1.52	7.33 $\pm$ 0.57	2	3
28	Khalki et al. (2010)	2.33 $\pm$ 1.15	4.67 $\pm$ 0.57	6.00 $\pm$ 0.00	2.33 $\pm$ 1.15	0.67 $\pm$ 1.15	16.00 $\pm$ 3.60	7.00 $\pm$ 1.00	2	3

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Table 5. (Continued)

Sr. No.	Authors	Criteria group I (Mean $\pm$ SD)	Criteria group II (Mean $\pm$ SD)	Criteria group III (Mean $\pm$ SD)	Criteria group IV (Mean $\pm$ SD)	Criteria group V (Mean $\pm$ SD)	Combined score (Mean $\pm$ SD)	Weighted score (Mean $\pm$ SD)	Initial category (Based on mean combined score)	Revised category (Based on mean weighted score)
29	Maheshwari et al. (2017)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	4.00 $\pm$ 1.00	2.67 $\pm$ 0.57	2.00 $\pm$ 0.00	17.67 $\pm$ 1.52	6.00 $\pm$ 1.00	2	3
30	Majumdar et al. (2017)	3.33 $\pm$ 1.15	4.33 $\pm$ 0.57	5.33 $\pm$ 1.15	2.67 $\pm$ 0.57	1.33 $\pm$ 0.57	17.00 $\pm$ 3.60	7.00 $\pm$ 1.00	2	3
31	Mokashi et al. (2014)	2.67 $\pm$ 0.57	5.00 $\pm$ 0.00	6.33 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	19.00 $\pm$ 0.00	7.33 $\pm$ 0.57	1	3
32	Panda et al. (1999)	1.33 $\pm$ 0.57	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	17.33 $\pm$ 0.57	7.00 $\pm$ 0.00	2	3
33	Petit et al. (1995)	3.67 $\pm$ 0.57	5.00 $\pm$ 0.00	5.67 $\pm$ 0.57	3.00 $\pm$ 0.00	1.33 $\pm$ 1.15	18.67 $\pm$ 2.30	7.33 $\pm$ 1.15	1	3
34	Poole et al. (2010)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	5.33 $\pm$ 0.57	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	19.33 $\pm$ 0.57	7.33 $\pm$ 0.57	1	3
35	Rao et al. (2015)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	2.67 $\pm$ 0.57	1.67 $\pm$ 0.57	19.33 $\pm$ 1.15	8.00 $\pm$ 0.00	1	1
36	Rao et al. (2016)	1.00 $\pm$ 0.00	5.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.33 $\pm$ 0.57	0.00 $\pm$ 0.00	11.33 $\pm$ 0.57	5.00 $\pm$ 0.00	3	3
37	Sharma (1986)	2.00 $\pm$ 0.00	4.33 $\pm$ 1.15	6.67 $\pm$ 0.57	2.33 $\pm$ 1.15	1.00 $\pm$ 1.00	16.33 $\pm$ 3.78	7.33 $\pm$ 1.15	2	3
38	Steels et al. (2011)	2.00 $\pm$ 1.00	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	18.00 $\pm$ 1.00	8.00 $\pm$ 0.00	1	1
39	Swaroop et al. (2014)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	2.67 $\pm$ 0.57	2.00 $\pm$ 0.00	19.67 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
40	Wankhede et al. (2016)	4.00 $\pm$ 0.00	5.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.00 $\pm$ 0.00	8.00 $\pm$ 0.00	1	1
41	Wilborn et al. (2010)	2.33 $\pm$ 1.15	5.00 $\pm$ 0.00	6.33 $\pm$ 0.57	2.67 $\pm$ 0.57	2.00 $\pm$ 0.00	18.33 $\pm$ 0.57	8.00 $\pm$ 0.00	1	1
42	Mowla et al.	1.00 $\pm$ 0.00	4.00 $\pm$ 0.00	6.33 $\pm$ 0.57	0.67 $\pm$ 0.57	0.00 $\pm$ 0.00	12.00 $\pm$ 0.00	7.00 $\pm$ 0.00	3	3

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Table 5. (Continued)

Sr. No.	Authors	Criteria group I (Mean $\pm$ SD)	Criteria group II (Mean $\pm$ SD)	Criteria group III (Mean $\pm$ SD)	Criteria group IV (Mean $\pm$ SD)	Criteria group V (Mean $\pm$ SD)	Combined score (Mean $\pm$ SD)	Weighted score (Mean $\pm$ SD)	Initial category (Based on mean combined score)	Revised category (Based on mean weighted score)
	(2009)									
43	Hamad et al. (2017)	0.00 $\pm$ 0.00	4.00 $\pm$ 0.00	3.00 $\pm$ 0.00	0.67 $\pm$ 1.16	0.00 $\pm$ 0.00	7.67 $\pm$ 1.16	4.00 $\pm$ 0.00	3	3
44	Wilborn et al. (2017)	3.00 $\pm$ 0.00	5.00 $\pm$ 0.00	7.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	20.00 $\pm$ 0.00	8.00 $\pm$ 0.00	1	1
45	Hamad et al. (2017)	0.00 $\pm$ 0.00	4.33 $\pm$ 0.56	3 $\pm$ 0	0.33 $\pm$ 0.58	0.00 $\pm$ 0.00	7.67 $\pm$ 1.15	4.00 $\pm$ 0.00	3	3

Data were represented as Mean $\pm$ SD.

Table 6. ToxR reliability assessment scores for individual *in-vitro* toxicity studies.

Sr. No.	Authors	Criteria group I (Mean $\pm$ SD)	Criteria group II (Mean $\pm$ SD)	Criteria group III (Mean $\pm$ SD)	Criteria group IV (Mean $\pm$ SD)	Criteria group V (Mean $\pm$ SD)	Combined score (Mean $\pm$ SD)	Weighted score (Mean $\pm$ SD)	Initial category (Numerical result)	Revised categories after checking red scores
1	Deshpande et al. (2017a)	4.00 $\pm$ 0.00	2.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	17.00 $\pm$ 0.00	6.00 $\pm$ 0.00	1	1
2	Deshpande et al. (2016c)	4.00 $\pm$ 0.00	2.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	17.00 $\pm$ 0.00	6.00 $\pm$ 0.00	1	1
3	Deshpande et al. (2017c)	4.00 $\pm$ 0.00	2.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	17.00 $\pm$ 0.00	6.00 $\pm$ 0.00	1	1
4	Flammang et al. (2004)	1.33 $\pm$ 1.15	3.00 $\pm$ 0.00	6.00 $\pm$ 0.00	3.00 $\pm$ 0.00	0.00 $\pm$ 0.00	13.33 $\pm$ 1.15	4.67 $\pm$ 0.57	2	3
5	Swaroop et al. (2014)	4.00 $\pm$ 0.00	2.33 $\pm$ 0.57	5.33 $\pm$ 1.15	3.00 $\pm$ 0.00	2.00 $\pm$ 0.00	16.67 $\pm$ 0.57	5.33 $\pm$ 1.15	1	3

Data were represented as Mean $\pm$ SD.

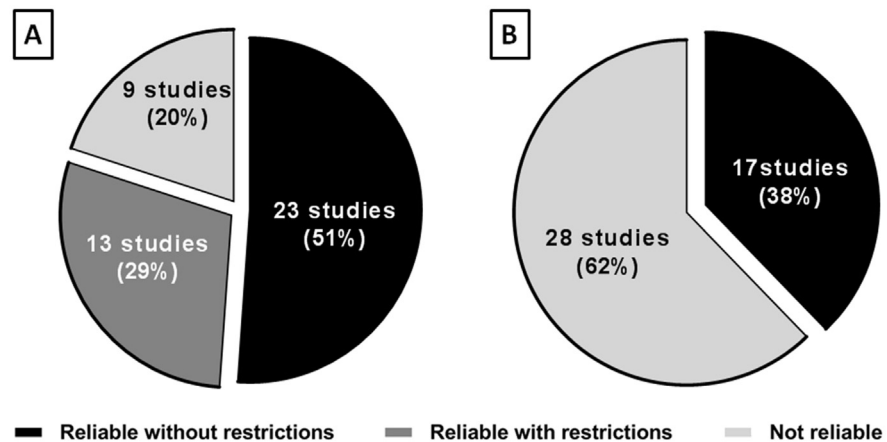


Fig. 2. Number of *in-vivo* studies in the combined score (A) and weighted score (B) categorization.

three studies found to be “Reliable Without Restriction” (60%) and two studies were “Not Reliable” (40%) (Fig. 3). Three studies were having the highest combined score as well as weighted score amongst five evaluated *in-vitro* studies [8, 33, 35].

3.4. The effects of identification of the test material based on the average combined score and weighted score

Univariate analysis showed that the identified category i.e., studies which have used identified study material or studies which have used isolated phytoconstituents, have significant difference ($p < 0.0001$) for average combined score (i.e., initial category score) and weighted score (i.e., revised category score) as compared to non-identified study material or studies which have been carried out on fenugreek seed extract or diet/feed.

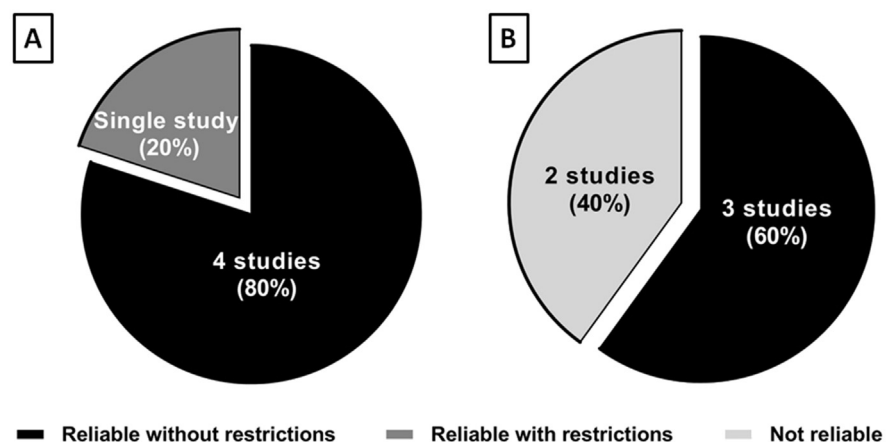


Fig. 3. Number of *in-vitro* studies in the combined score (A) and weighted score (B) categorization.

3.5. The effect of compliance with guidelines based on the average combined score and weighted score

The toxicity studies which have been conducted according to the guidelines such as OECD (Organization for Economic Co-operation and Development) or ICH-GCP (International Conference on Harmonization-Good Clinical Practice) have a significant difference ($p < 0.05$) for an average combined score and a weighted score of scores as compared to studies where guidelines are not followed.

3.6. The correlations of various criteria groups with an average combined score

The Pearson correlation between an average combined score and scores for the groups I (i.e., test substance identification), III (i.e., study design description) and V (i.e., the plausibility of study design and results) were found to be 0.875, 0.734, and 0.905 respectively. The Kendall rank correlation coefficient (Kendall's tau-b) of criteria for the groups I, III, and V with the average combined score was found to be 0.764, 0.551, and 0.752 respectively. The average of total score of group I, III, and V has a significant effect ($p < 0.05$) on average of combined score.

3.7. The outcome of fenugreek seed toxicological studies that are categorized as “Reliable without restrictions.”

The outcome from the studies categorized as “Reliable without restrictions” is presented in Table 7. All these seventeen studies used identified study material (standardized fenugreek seed extract with identified marker compounds) for evaluation of fenugreek seed toxicity.

Table 7. The outcome of fenugreek seed toxicological studies that are categorized as ‘Reliable without restrictions’.

Sr. No.	Authors	Outcomes
1.	Aswar et al. (2009)	The lack of abortifacient activity or teratogenicity confirms the <i>safety</i> of trigonelline during pregnancy in rats
2.	Deshpande et al. (2017a)	The furostanol saponin glycoside based standardized fenugreek seed extract (Fenu-FG) was found <i>safe</i> during preclinical safety assessments
3.	Deshpande et al. (2016a)	The prenatal oral exposure of Fenu-FG was <i>devoid</i> of maternal or developmental <i>fetotoxicity</i> or <i>teratogenicity</i>
4.	Deshpande et al. (2016b)	Oral exposure of low molecular weight galactomannans based standardized fenugreek seed extract (LMWGAL-TF) during the prenatal period <i>did not</i> induce significant maternal and embryo-fetal <i>toxicity</i>
5.	Deshpande et al. (2017b)	<i>None</i> of the Glycoside-based standardized fenugreek seed extract (SFSE-G)-treated groups showed maternal and embryo-fetal <i>toxicity</i>

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Table 7. (Continued)

Sr. No.	Authors	Outcomes
6.	Deshpande et al. (2016c)	SFSE-G was found <i>safe</i> for acute and sub-chronic administration in rats with no mutagenic potential
7.	Deshpande et al. (2017c)	IDM01 (the botanical composition of 4-hydroxyisoleucine- and trigonelline-based standardized fenugreek) was found <i>safe</i> during preclinical acute and sub-chronic toxicity in rats without mutagenicity or genotoxicity
8.	Deshpande et al. (2016d)	LMWGAL-TF was found <i>safe</i> during acute and sub-chronic toxicity studies in rats with no mutagenicity
9.	Folwarczna et al. (2014b)	Administration of trigonelline <i>did not affect</i> the investigated parameters in non-ovariectomized rats
10.	Kandhare et al. (2015)	SFSE-G was found <i>safe</i> for acute and subacute administration in rats
11.	Kandhare et al. (2016a)	Vicenin-1 was found <i>safe</i> for acute and subacute administration in rats
12.	Rao et al. (2015)	<i>T. foenum-graecum</i> seed extract is a <i>well-tolerated</i> and is an effective botanical medicine for use in the support of sexual function of pre-menopausal women
13.	Steels et al. (2011)	There were <i>no adverse events</i> recorded during the clinical trial. The Testofen, a standardized <i>Trigonella foenum-graecum</i> (Fenugreek) extract was <i>well tolerated</i> .
14.	Swaroop et al. (2014)	After extensive <i>in-vitro</i> and <i>in-vivo</i> safety and efficacy studies it has been concluded that novel fenugreek extract (FE), Fenfuro TM is <i>safe</i> and effective in treating type 2 diabetes.
15.	Wankhede et al. (2016)	The Fenu-FG supplementation was found to be <i>safe</i> and <i>well-tolerated</i> .
16.	Wilborn et al. (2010)	<i>T. foenum-graecum</i> appears <i>safe</i> when taken over an 8-week time period
17.	Wilborn et al. (2017)	IBPR was found to be safe and well tolerated

4. Discussion

There are a large number of clinical and preclinical published evidence available on the toxicological and safety profile of fenugreek seed. However, several considerations such as nature of study material, variable experimental designs, conflicting results, etc. affect the safety outcomes. There are few reviews available which summarize the toxicological outcomes of these fenugreek toxicity studies [9, 10]. However, the utility of these reviews for risk assessment is limited as they have summarized results without any consideration of critical assessment of the quality of the studies. Hence, in the present investigation three-independent raters systematically evaluated available evidence of fenugreek seed toxicological studies by using the ToxRTool. This approach consists of (1) identification or characterization of the fenugreek seed or isolated substance from it, (2) compositional analysis of the fenugreek seed feed, (3) study design carried out according to the international guidelines, and (4) good quality of study results or adverse event reporting in toxicology studies. The outcome of the present study provided a transparent and comprehensive assessment of *in-vivo* and *in-vitro* published evidence on fenugreek seed toxicological studies with a critical assessment using the ToxRTool.

In the present study, ToxRTool was employed to provide a transparent method for risk assessment of the published evidence. Evaluation of published toxicity studies using ToxRTool provided important information about the critical components of

the evaluation of toxicological data reliability and possible sources of heterogeneity which affect the outcome of the evaluation. Animal feeding studies are widely accepted for assessment of risks for human food consumption thus, a thorough search of the literature has been conducted to identify fenugreek seed toxicological studies as many as possible.

The chemical structure of the compound is a key determinant of the potential of toxicity [60]. Thus, chemical characterization of food chemical or food component needs to be an initial step towards its toxicological evaluation. The availability of technical information of a structure of the compound can provide valuable insight, for instance, irritability, bioaccumulation or any inhalation hazard, etc. [61]. The technical information includes any common names, trade names, Chemical Abstracts Service (CAS) number, chemical name, chemical composition, degree of purity, solubility, physical form, presence of any known impurities along with its nature, physicochemical properties such as pH, pKa, melting point, boiling point, vapour pressure and partition coefficient and stability. In addition to technical information, description of the manufacturing process, the presence of any complex mixtures and reliable analytical methods for detection or quantification of the test substance may provide important basis for reliable toxicological information [61]. Thus, the inclusion of comprehensive and clear information of test substance needs to be ensured for reliable safety information.

In the present study, the toxicological studies in which such comprehensive technical information of extract (either aqueous, hydro-alcoholic, alcoholic or petroleum ether) were not identified, showed low combined score and fell under category 3, i.e., “Not Reliable”. On the other hand, the toxicological studies, in which clear identification of composition of fenugreek seeds was reported, that studies showed higher combined score (≥ 18) and fell in the category ‘1’ i.e., “Reliable Without Restrictions”. These were further supported by the correlation analyses (Pearson as well as Kendall rank correlation coefficient), i.e., criteria group I (i.e., test substance identification) showed good correlation with combined scores. These results strongly delineate the importance of identification of the test substance on the quality and reliability of toxicity study.

The implementation of animal-based methods for determination of hazard is an important component of human risk assessment [62]. Hence, the selection of animal species for determination of toxicity is an important aspect for clinical toxicological relevance. Moreover, the practicality of animal model such as ease of assessment, availability of background data, ease of use, etc., also played a significant role in toxicity evaluation. Furthermore, it has been reported that obtaining maximum information by utilizing a minimum number of animals is generally emphasized during toxicological evaluations [61]. These can be achieved by using either sex, implementing multiple species (such as mice and rats), the use of higher and multiple

doses which are higher than anticipated human exposure and administration of dose over a long duration which covers the complete life cycle. These careful considerations affect the toxicological outcomes of test compounds. In the present review, the selected fenugreek seed toxicity studies have used various species such as albino and Swiss mice, Wistar and Sprague-Dawley rats, goat, fish, New Zealand white rabbits, healthy human volunteers, etc. Most of the toxicological studies reported the test organism characterization well. Thus, the findings from ToxRTool showed that total score of group II (i.e., test organism characterization) did not have any significant effect on average combined scores or reliability category of the study.

The minimum number of animals per dose group is another important aspect of quality animal study concerning statistical analysis. The OECD guideline 414 [63] recommends consideration for usage of a sufficient number of animals. Furthermore, the quality of the outcome data depends on the various animal-related factors including health of the animal stock, variability of animals, environmental condition of the laboratory and animal house, quality of diet, nutritional adequacy of diet, and the qualifications and experience of the staff [61]. The finding of the present study showed a good correlation (Pearson) criteria group III (i.e., study design description) with a combined score and a weighted score of ToxRTool.

The repeated dose toxicity studies are undertaken with an objective to determine any possible adverse effect of the test compound, on long-term (subacute, sub-chronic or chronic) toxicities and to determine the no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL). NOAEL and LOAEL form an important base for determination of maximum tolerable daily intakes and dose-response relationships [64]. During repeated dose toxicity study, test substance administration in the form of diet/feed is common because of its relevance to oral intake in humans. Additionally, administration of test substance in drinking water is correlated well with use as beverages. However, administration via diet/feed or drinking water has the limitation that the exact dose and quantification of administered test substance can't be determined. Furthermore, administration of nutritionally unbalanced diets may result in adverse event which may conclude in negative toxicological outcomes [65, 66]. In the present study, five studies used the fenugreek seed in the form of diet [23, 27, 38, 41, 55]. These studies did not report values for nutritional analysis of diet or food intake by the animal or subject and therefore may not yield reliable toxicology information. Thus, administration by oral gavage provides advantages over diet or drinking water. The results of the present study support the administration of study material by oral gavage over diet. In line with this notion, all 5 studies with diet route of administration were categorized under the category '3' and supported non-reliability of these studies.

The present study also considered "quality of study design" as an important aspect of the toxicological study. Additionally, the toxicology study outcomes were greatly

influenced by the extent of details and quality of data analysis. Hence, selection of robust and appropriate statistical analysis methods is important for the toxicological study outcomes [61] and is considered in the international guidelines. Therefore, studies which have been conducted according to international guidelines for toxicology are expected to have robust experimental design and data analysis. This notion is supported by the results of the present study where studies with good quality of study-design are categorised as category 1 ('Reliable Without Restriction') or 2 ('Reliable with Restriction'). Conversely, the studies that are not conducted as per principals of GLP (Good laboratory practice) and did not follow international guidelines were assigned to the category 3 ('Not Reliable'). Furthermore, scores of criteria group V (i.e., the plausibility of study design and results) have a good Pearson correlation (0.905) with the combined score and also have a significant effect ($p < 0.05$) on combined score.

Animal-based *in-vivo* toxicological evaluation is a good predictor of hazards to human health [67]. However, it has limited predictability for carcinogenicity and developmental toxicity potential due to a limited number of known carcinogens and teratogens with their species-specific responses of animals [68]. Therefore, *in-vitro* mutagenicity (Ames test) study (OECD No. 471) can provide the dose-response data for hazard identification and risk assessment for carcinogenicity and mutagenicity [67]. Furthermore, *in-vitro* genotoxicity (chromosomal aberrations test) (OECD No. 473) is a reliable and validated method for reproductive and developmental toxicity testing. In the present study, five toxicological studies on fenugreek seed extract investigated *in-vitro* mutagenicity and genotoxicity. 3 out of the 5 *in-vitro* studies were conducted according to international guidelines and were found to fall under category 1, i.e. 'Reliable Without Restriction' [8, 33, 35].

The reliability of toxicological study outcomes largely depends on integrity and quality conduct of the studies [61]. Over the last two decades, toxicological studies which were carried out according to the international guidelines such as WHO protocol, OECD, or ICH-GCP have much reassurance for study outcomes. The guidelines help to ensure good experimentation and provide quality and consistent outcome data. Nevertheless, other toxicological studies which have not been conducted with such guidelines also provided useful results. However, such studies need to be considered with caution for toxicity risk assessment. In this regard, the assessments based on ToxRTool can be valuable. Amongst the toxicological studies that were conducted as per guidelines, 17 (38%) of studies were considered as 'Reliable Without Restrictions' and remaining 28 (62%) studies were considered as 'Not Reliable'. The present study found that fenugreek toxicity publications [6, 8, 20, 25, 29, 30, 32, 33, 34, 35, 40, 44, 52, 53] that are conducted according to the international guidelines produce more reliable results.

The present study utilized the ToxRTool tool to assess the reliability and relevance of published toxicological studies by three raters. During such assessments, heterogeneity can result from differences in raters' experience and their interpretation of criteria [69]. Therefore, we conducted an inter-rater reliability analysis to determine the extent to which variation exists between different raters by using validated instrument to obtain similar outcomes. Inter-rater reliability also ensures that the stability of generated data resulted in the form of observational analysis. In this study, three independent reviewers (PAT, ADK, PW) assessed individual fenugreek seed toxicological studies by using ToxRTool. Inter-rater consistency reliability for the three raters was 0.920, i.e., 92.0 % agreement between the raters for a combined score. If rating would have been done by a single rater, then the interclass correlation coefficient for the single measure would be 0.794. Therefore, in the present study use of three raters for scoring instead of single rater resulted in a 12.6% increase in correlation in combined scores. Similarly, for weighted score, a single rater would have resulted in an interclass correlation coefficient of 0.724, whereas three raters provided an additional 16.3% correlation amount weighted scores. Therefore, the average combined score and the average weighted score was used for further analysis.

In the present ToxR evaluation, analysis of conclusions of all the toxicological studies that were identified as Category 1 ('Reliable without restrictions') suggested a broad margin of safety for long-term duration (Table 7). All these studies utilized standardized test compound from fenugreek seed with well-identified marker (Table 7). Hence, the results of the present study emphasize the need to use the well-identified compounds in toxicological studies for reliable outcome.

There are some limitations existing in the present investigation and should be considered into account while interpreting the results of present findings. Firstly, the study did not analyze the published toxicological literature on parts of fenugreek plant other than seeds. Furthermore, any unavailable or missing information, or publication bias in reporting toxicological studies have not been taken into consideration.

5. Conclusions

The comprehensive analysis of fenugreek seed toxicity studies using ToxRTool suggested a systematic approach that was found useful to evaluate the quality of published toxicological studies for health risk assessment. In the present study, toxicological publications on fenugreek seeds that are categorized as 'Reliable without restrictions' can be considered for toxicological risk assessment with reasonable certainty. The toxicological studies that are categorized as 'Reliable with restrictions' can be considered for toxicological risk analysis with caution especially

concerning identification (characterization) of the compound. The studies categorized under category 3 (Not reliable) may not be useful for toxicological risk assessments.

Declarations

Author contribution statement

Amit Kandhare: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Prasad Thakurdesai: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Pralhad Wangikar: Performed the experiments; Analyzed and interpreted the data.

Subhash Bodhankar: Analyzed and interpreted the data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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